Evaluation of Challenges to the Ubiquitous Nature of Chromatography

A thesis submitted to University College London

for the degree of Doctor of Engineering

by

Richard Tran

The Advanced Centre for Biochemical Engineering

Department of Biochemical Engineering

University College London

Torrington Place

London WC1E 7JE

Abstract

Packed bed chromatography is the workhorse of the majority of downstream purification processes used for the manufacture of biopharmaceutical therapeutics. This high dependence upon chromatography has lead to concerns being raised regarding the manufacturing costs and also the potential constraints on plant productivity imposed by packed bed processes, particularly in light of advances seen in upstream operations. This has not unsurprisingly generated a significant degree of discussion amongst the bioprocessing community on how best to deal with these challenges. Amongst the proposed strategies, is the adoption of what may be termed "alternative" bioseparation techniques, which may potentially offer higher processing capacities at a lower cost.

In this study a Multi-Attribute Decision Making MADM) based framework was used to evaluate these bioseparation techniques being considered as potential alternatives to packed bed chromatography. This evaluation included consideration of a wide range of process characteristics, beyond just performance and cost related attributes, but also considering areas such as the ease of process development, operation and scalability. The use of this framework not only allows the most promising technologies to be identified, but manipulation of the non-deterministic outputs of this framework permitted indications to be obtained as to the most productive directions for further technology development.

Using the indicators provided by this framework, experimental studies were carried out in order to study the performance of these most promising alternatives, when used as part of a whole downstream processing train. These studies yielded information upon the interactions between these different bioseparation technologies and allowed

the impact on process productivity and process economy to be evaluated.

The collective findings from this study reinforce the generally held opinion that none of these alternative bioseparation techniques can currently be considered the key to overcoming the challenges faced by downstream processing. Indeed evaluations of the techniques imply that the ubiquitous nature of packed bed chromatography is likely to remain for the foreseeable future. However the tools which have been developed allow for a rational explanation of what exact factors are the barriers to adoption for these techniques, and as a consequence provide guidance as to the areas in which future development is most required.

Declaration of Authorship

I, Richard Tran, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Contents

1 Purpose of Study **1**

2 Is Chromatography Indispensable? **5**

2.1 Introduction 5

2.1.1 Monoclonal Antibody Manufacturing 5

2.1.2 Protein A and Platform Processes 9

2.1.3 The Constraints of Packed Bed Chromatography 22

2.2 Future Challenges for Downstream Purification 34

2.2.1 The Multi-ton Manufacturing Process 34

2.2.2 Flexible Multi-Product Facilities 38

2.2.3 Industry Trends 42

2.3 Addressing The Incoming Challenges 45

2.3.1 Improving Current Technologies 45

2.3.2 Disposable Technologies 48

2.3.3 Process Intensification and Continuous Processing 49

2.3.4 Alternative Bioseparation Technologies 54

3 Alternative Bioseparation Techniques **57**

3.1 Introduction 57

3.2 Bulk Separation Techniques 59

3.2.1 Aqueous Two Phase Extraction 60

3.2.2 Affinity Precipitation 68

3.2.3 Three Phase Partitioning 76

3.2.4 Macroligand Facilitated Three Phase Partitioning 84

3.2.5 Crystallisation 89
3.3 Field-based Separations 99
3.3.1 High Performance Tangential Flow Filtration 99
3.3.2 Controlled Shear Affinity Filtration 107
3.4 Adsorptive Separations 115
3.4.1 Expanded Bed Adsoption Chromatography 115
3.4.2 Monoliths 123
3.4.3 Membrane Chromatography 131
3.5 Conclusions 140

4 Changing Manufacturing Paradigms in Downstream Processing 143
4.1 Abstract 143
4.2 Introduction 144
4.3 Materials and Methods 147
4.3.1 Bioseparation Alternatives Survey I 148
4.3.2 Bioseparation Alternatives Survey II 149
4.4 Results and Discussion 150
4.4.1 Confidence Levels and Intervals 150
4.4.2 Importance Weighting 154
4.4.3 Organisation Types 160
4.4.4 Products of Manufacture 164
4.4.5 Alternative Technologies Used 164
4.4.6 State of Chromatography 173
4.4.7 Alternative Techniques Evaluated 175
4.4.8 Future Challenges 182
4.5 Conclusions 187

5 A Methodology for the Comparative Evaluation of Alternative Bioseparation Technologies 190
5.1 Abstract 190
5.2 Introduction 191

5.3 Materials and Methods . . . 196
5.3.1 Multi-Attribute Decision Making (MADM) . . . 196
5.3.2 Attributes . . . 197
5.3.3 Importance Weightings and Attribute Ratings . . . 200
5.3.4 MADM Scoring Technique . . . 203
5.3.5 Monte Carlo Simulations . . . 204
5.3.6 Attribute Importance Weights . . . 205
5.3.7 Attribute Ratings for Techniques . . . 207
5.3.8 Bioseparation Techniques . . . 212
5.3.9 Analysis Scenario . . . 212
5.4 Results and Discussion . . . 215
5.4.1 Attribute Group Scores . . . 215
5.4.2 Overall "Industrial Attractiveness" Scores . . . 219
5.4.3 Accounting for Process Trade-offs . . . 220
5.4.4 Non-deterministic Analysis . . . 223
5.4.5 Sensitivity and Scenario Analysis . . . 228
5.4.6 Head to Head Comparisons of ATPE and HPTFF . . . 229
5.4.7 Developing the Industrial Attractiveness of ATPE and HPTFF . 235
5.4.8 Further Improving the Industrial Attractiveness of ATPE and HPTFF . . . 242
5.4.9 Sensitivity Analysis to Inform Business Related Decisions . . . 247
5.5 Conclusions . . . 247

6 Integration of Alternative Bioseparations Techniques into a mAb Purification Platform Process - Part I 252

6.1 Abstract . . . 252
6.2 Introduction . . . 253
6.2.1 The Base Case . . . 256
6.2.2 Aqueous Two Phase Extraction . . . 257
6.2.3 High Performance Tangential Flow Filtration . . . 258

6.2.4 Process Feed . . . 259
6.2.5 Process Parameters . . . 260
6.3 Materials and Methods . . . 261
6.3.1 Feed Material . . . 261
6.3.2 Chemicals . . . 262
6.3.3 Base Case Chromatography Process . . . 262
6.3.4 Aqueous Two Phase Extraction . . . 264
6.3.5 High Performance Tangential Flow Filtration . . . 265
6.3.6 Analytics . . . 269
6.4 Results and Discussion . . . 270
6.4.1 Base Case Process . . . 270
6.5 ATPE Forward Extraction Development . . . 275
6.5.1 ATPE Forward Extraction . . . 275
6.5.2 Impact of Order of Powder Addition . . . 276
6.5.3 Interfacial Precipitate and Mass Balance . . . 278
6.5.4 Differences in Forward Extraction System Performance . . . 279
6.6 ATPE Back Extraction Development . . . 280
6.6.1 Decreased Phosphate Concentration . . . 282
6.6.2 Decreased System pH . . . 286
6.6.3 Alternative Anion . . . 289
6.6.4 Increasing PEG Concentration and Multi-stage Back Extraction 292
6.6.5 Decreasing NaCl Concentration . . . 302
6.6.6 Single Stage Back Extraction . . . 311
6.6.7 Alternative ATPE System . . . 313
6.6.8 Summary of ATPE Development . . . 314
6.7 HPTFF Development . . . 317
6.7.1 Choice of membrane MWCO . . . 318
6.7.2 Uncharged Membrane Characterisation . . . 320
6.7.3 Purification Factor and Yield of Ultrafiltration with Uncharged Membrane . . . 326

6.7.4 Normalised Water Permeability (NWP) . . . 330
6.7.5 Charged Membrane Characterisation . . . 332
6.7.6 Purification Factor and Yield of Ultrafiltration with Charged Membrane . . . 337
6.7.7 Normalised Water Permeability of Charged Membrane . . . 339
6.8 Conclusions . . . 340

7 Integration of Alternative Bioseparations Techniques into a mAb Purification Platform Process - Part II 344

7.1 Abstract . . . 344
7.2 Introduction . . . 345
7.3 Methods and Materials . . . 347
7.4 Results and Discussion . . . 348
7.4.1 Process Train 2 . . . 348
7.4.2 Process Train 2A . . . 361
7.4.3 Process Train 3 . . . 368
7.4.4 Process Train 4 . . . 379
7.4.5 Process Train 5 . . . 393
7.5 Conclusions . . . 399

8 Process Economy Evaluation of Alternative Techniques 405

8.1 Abstract . . . 405
8.2 Introduction . . . 406
8.3 Materials and Methods . . . 408
8.3.1 Process Models . . . 409
8.3.2 Process Economy Metrics . . . 411
8.4 Results and Discussions . . . 414
8.4.1 Summary of Process Performance . . . 414
8.4.2 Process Productivity . . . 415
8.4.3 Cost of Goods . . . 418
8.4.4 Specific Productivity . . . 420

8.4.5 Potential Performance . . . 423
8.4.6 Process Economy in Light of Increasing Product Titre . . . 427
8.5 Conclusions . . . 432

9 Conclusions **435**
9.1 Constraints of Platform Processes and the Need for Alternative Technologies . . . 435
9.2 Theoretical Evaluation of Alternative Technologies . . . 436
9.3 Practical Evaluation of Alternative Technologies . . . 438
9.4 The Future Potential of Alternative Technologies . . . 439
9.5 Advantages of the Platform Approach . . . 440
9.6 Sustainability of Current MAb Manufacturing Paradigms . . . 440

10 Future Work **442**
10.1 Process Development . . . 442
10.2 Process Robustness . . . 443
10.3 Increased Product Titre . . . 444
10.4 Cost of Development . . . 445
10.5 Alternative Bioproducts . . . 445

11 Appendix A: GE Healthcare Letter **447**

12 Appendix B: Patent Application **449**
12.1 Introduction . . . 449
12.2 Abstract . . . 450
12.3 Field of the Invention . . . 450
12.4 Background of Invention . . . 450
12.5 Summary of the Invention . . . 454
12.6 Brief Description of the Drawings . . . 455
12.7 Detailed Description of the Invention . . . 456
12.7.1 Definitions . . . 456
12.7.2 Method for Purification of a Target Protein . . . 457

12.8 Examples 467
12.8.1 Example 1 - Primary Capture and Purification of MAb using ATPE assisted precipitation 468
12.9 References Cited 475
12.10 Claims 476

13 Appendix C: Published Chapters **479**

Bibliography **480**

Chapter 1

Purpose of Study

In light of advances seen in upstream technologies, concerns have been raised regarding the potential cost and productivity issues associated with downstream processing, which may be encountered as a result of its high dependence on the use of packed bed chromatography. One of the methods through which it has been proposed that these issues may be addressed is through the use of alternative bioseparation techniques, to augment and potentially replace conventional chromatographic operations. It has been argued that in doing so it may be possible to impose a paradigm shift in bioprocessing, diminishing the reliance on packed bed operations and therefore enabling the cost and productivity concerns associated with current manufacturing platforms, in which conventional chromatography is so ubiquitous, to be overcome.

Given the increasing pressures on speed to market for new biopharmaceutical products, the decision faced by bioprocess engineers is whether to devote precious time and resources to the development of these alternatives. Such a decision will ultimately depend upon the potential advantages which may be conferred through the utilisation of these techniques, possibly in place of packed bed chromatography, and whether such advantages justify the additional development effort which is required.

To date, evaluations of these technologies have been limited to qualitative assessments of the potential advantages which may be conferred through their utilisation. Furthermore, data regarding the performance of these techniques is generally restricted to cases in which they have been studied in isolation rather than a part

of a whole process sequence. There is therefore insufficient information available to determine true effectiveness of these techniques in enabling the potential issues of cost and productivity, associated with downstream processing due to its high reliance on packed bed chromatography, to be overcome.

The overall aim of this thesis is to therefore to address this knowledge gap, by presenting a general quantitative evaluation of these alternative techniques. Doing so will not only allow the most industrially applicable alternative techniques to be identified, but will also provide clear insights regarding the genuine feasibility of adopting them into biomanufacturing processes to be gained. Such an assessment will need to account not only for the advantages, but also importantly the key limitations and drawbacks which may be associated with their utilisation.

To meet this aim, a combination of both experimental studies and process models will be used in order to assess the alternatives in terms of key processing aspects. By doing so it will be possible to quantify the advantages these techniques have over packed bed chromatography in terms of tangible biomanufacturing metrics, including not only technical process parameters such as the process yield and purification factor, but also process economy related elements such as the cost of goods and process throughput. Quantification in this manner will also provide insight as to the areas in which the alternatives require most development in order to ensure their effectiveness in tackling the issues of cost and productivity associated with downstream processing. As previously alluded to, in order to ensure the validity of the results of this analysis, the evaluation of these technologies must take into account their performance from a whole bioprocess perspective and also when operated at scale.

Given the expansive scope of such an undertaking, the aforementioned evaluation will be broken down into a number of different stages. Initially a review of separation techniques, currently being considered as potential alternatives to packed bed chromatography, will be performed. The aim of this will be to gain a deeper understanding of what these techniques actually are, the mechanisms of separation that they utilise and the ways in which they may be operated. Such understanding will be a prerequisite for any detailed evaluation of these technologies.

Following on from this, a survey of biomanufacturers will be performed in order to gain insight as to the alternative techniques which are currently already being used in large scale processing or are currently being actively investigated. This will provide information regarding the technical feasibility of using these technologies at scale, as well as providing an impression of the alternatives which currently have the highest popularity. An additional goal for this survey is to gain quantitative data on the level of value bioprocess engineers place on different process characteristics. This information will be vital when it comes evaluating the alternative techniques and balancing the trade-offs between different process attributes.

Using the data from both the review of alternatives and the results from the survey, the aim is to develop a process modelling tool which will enable the industrial attractiveness of bioseparation techniques to be determined. Using such a tool, it will be possible to identify the alternatives which show the most promise for incorporation into large scale biomanufacturing processes.

Once these techniques have been identified, the aim then would be to study them in greater depth, by applying them to an actual purification process. Experimental studies will be performed in order to assess the performance of these alternatives when used as part of a whole process sequence. Comparison of the performance of process trains incorporating the use of alternative techniques against a more conventional downstream processing sequence will allow a quantitive evaluation of the precise potential of these techniques.

The final step will then be to weigh the advantages in terms of process costs and productivity which may be conferred through the utilisation of these techniques against any associated drawbacks. These two process economy related metrics are the primary drivers for movement away from the currently high dependence upon packed bed chromatography. Using data gained from the experimental studies, process models will therefore be generated and used to calculate the improvements, if any, in process productivity and the cost of goods gained from using alternative bioseparation techniques.

The hope is that the cumulative results obtained from these studies will allow

conclusions to be drawn as to the potential challenge these techniques may pose to the currently pervasive nature of packed bed chromatography in downstream processing, particularly in the field of monoclonal antibody purification, where the concerns regarding process productivity and cost are at the forefront. This will help to inform decisions as to whether to pursue their development, and ultimately adoption in place of packed bed chromatography, into biomanufacturing processes.

Chapter 2

Is Chromatography Indispensable?

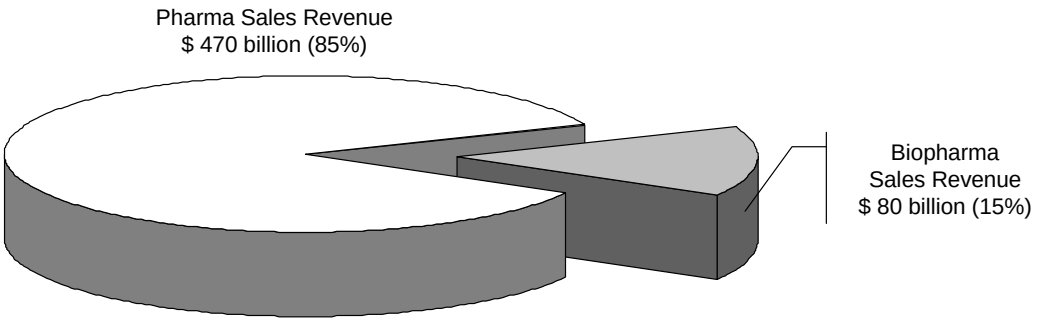
2.1 Introduction

Packed bed chromatography is the workhorse of the majority of downstream purification processes used for the manufacture of biopharmaceutical therapeutics. It's high associated resolving power makes it an ideal unit operation to enable the characteristically high purity requirements of bio-therapeutic drugs to be met. Nowhere is this high dependence upon packed bed chromatography more clearly illustrated than when dealing with the purification of monoclonal antibodies (mAbs).

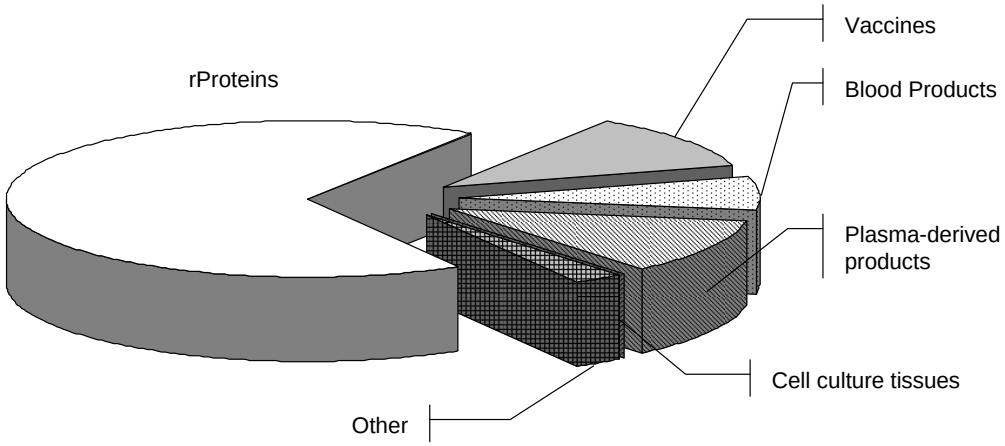
2.1.1 Monoclonal Antibody Manufacturing

The current international market for biopharmaceutical recombinant proteins (rProteins) is estimated to be worth approximately $50 billion.[1] This market is segmented into a number of different classes, comprising products such as interferons, insulins, hormones, blood factors and monoclonal antibodies amongst others. Figure 2.1 shows an approximate breakdown of the biopharmaceuticals markets from which the predominant nature of rProteins in terms of sales revenue and number of products can clearly be seen. The size of this rProtein market had more than doubled by 2009.

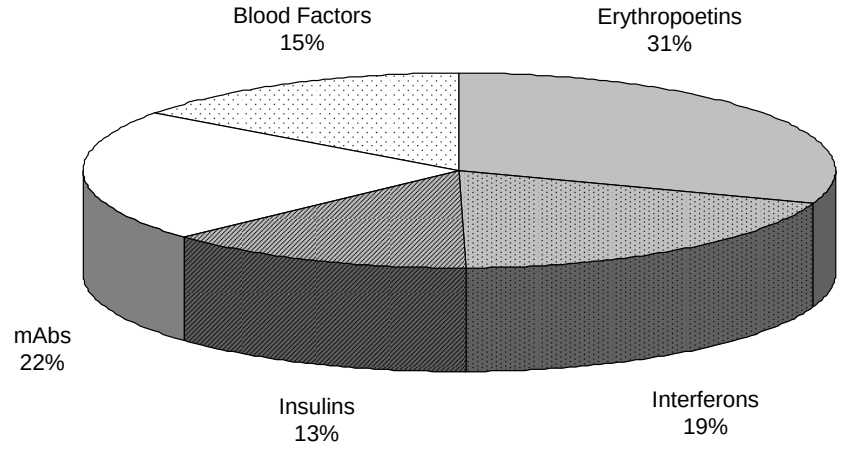
This increase in the size of the rProtein market is being powered by the dynamic pipeline of new products in development worldwide. Figure 2.2 shows the number of biopharmaceutical drugs in development in the United States of America since 2004,



(a) Sales revenue of pharma and biopharma products in 2004



(b) Biopharma sales revenue by product class in 2004



(c) Sales revenue of Blockbuster class rProtein products in 2004

Figure 2.1: Charts showing breakdown of the Biopharmaceutical market in terms of sales revenue generated

Data taken from Jacobi et al 2007 [1]

categorised by drug type. From this it can be seen that there is a very healthy number of rProtein drugs (including mAbs growth factors, interferons and interleukins) currently in development, a number of which has been seen to increase dramatically over the past 4 to 5 years, from 124 in 2004 to 284 in 2008. However, from Figure 2.2, it can be seen that this rise can almost be entirely attributed to the increase in the number of mAb drug candidates currently in development.

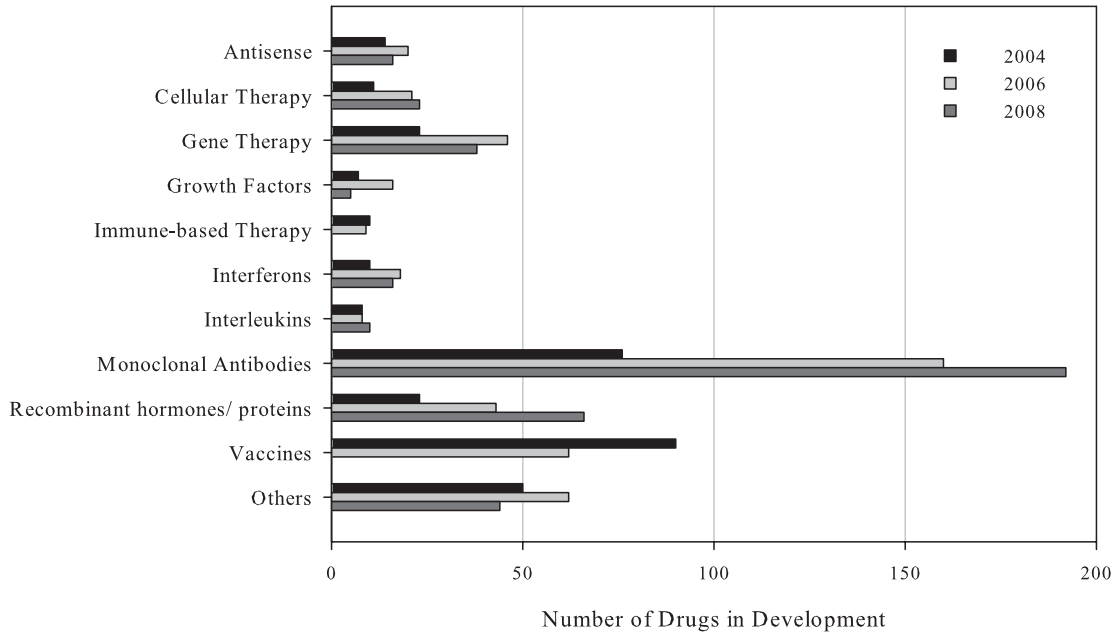


Figure 2.2: Breakdown of drugs in development pipelines
Taken from Source: PhrMA

The focus by biopharmaceutical developers on mAb based therapeutics has caused a shift in the rProteins market, away from previously predominant cytokine products (2.1(c)) and towards monoclonal antibodies. As of late 2004, 26 antibody-based therapeutic drugs had been approved in the United States and the European Union with combined annual sales thought to exceed $9 billion. By the end of 2006, 7 out of the 23 protein therapeutics which reached blockbuster status in the US (sales of greater than $1.1 billion) were monoclonal antibodies.[2, 3]. Given this growing value of the mAb therapeutic market, it is little wonder that the trends seen in the US (Figure 2.2) are reflected worldwide, with approximately 500 more mAb drug candidates thought to be currently in development by companies across the globe.[4]

By 2009, it was estimated that mAbs would make up between 30% and 50% of the total therapeutic protein market, representing a value of somewhere between $20 and $60 billion.[1]

The large number of candidates being developed and gaining regulatory approval, coupled with the relatively large dosage requirements associated with mAb products, means that not only must biomanufacturers generate more product than ever before, but they must also do so economically and without compromising product quality or safety.[1, 5] As a result manufacturing capacity, which is a finite quantity, becomes a vital constraint. An organisation may gain approval for three new mAb products each with a demand of 100kg/yr. However if their current manufacturing facility only has the capacity to produce 200kg of mAb per year, then product demands cannot be met and the organisation stands to lose significant revenues either through loss of market share, or via opportunity costs. Given that a new manufacturing facility may cost anywhere between $200-$400 million, and take up to 5 years to come on-line,[6] it is not feasible for biomanufacturers to adopt an approach whereby manufacturing capacity is added as it is needed. Instead, the challenge for process engineers is to find ways of maximising the productivity of their existing facilities whilst controlling the associated costs.

Technological developments upstream have gone some way towards addressing the previous challenges. Advances in mammalian cell culture technology, such as the development of more efficient expression systems along with improved media formulations and feeding strategies, have resulted in typical mAb titres rising from tenths of a gram per litre, up to 10 grams per litre, over the past decade.[7] This has been complimented by an increase in the fermentation capacity of manufacturing facilities worldwide. In 2000, the rProtein Enbrel® (etanercept), a soluble receptor for tumor necrosis factor, had become so popular that supply was not sufficient to meet demands. As a result, Immunex, the drug manufacturer was forced to place patients on waiting lists whilst fresh supplies were produced.[8] This lead, at the time, to the commonly held opinion that the biopharmaceutical industry was facing a shortfall in manufacturing capacity and for many Enbrel® was a sign of things to come. As a

direct response to this, a large building boom in manufacturing facilities occurred at the start of the new millennium. As it turned out, Enbrel® was actually an exceptional, rather than typical case, and as such the additional fermenter capacity was not as necessary as once thought. Nevertheless the result of this building boom is that companies now have access to bioreactors with volumes of up to 25,000L. The combination of high expressing cell cultures and large culture volumes means that it is potentially possible for such companies to produce as much as 250kg of mAb in a single bioreactor. This is almost 50 times the amount of mAb produced in a single batch from currently licensed monoclonal antibody processes.[9] As a result the focus has inevitably shifted downstream where technological advances have been much more incremental.

2.1.2 Protein A and Platform Processes

The purification of mAbs is currently firmly based upon the utilisation of a process platform built around the use of Protein A affinity chromatography.[10, 11] Protein A itself is a naturally occurring protein found anchored in the cell wall of *Staphylococcus aureus*.[12] Because of its origin, protein A displays specific affinity interactions with the Fc region of immunoglobulins, including immunoglobulin G (IgG). The interaction between the protein A molecule and the IgG, are thought to comprise a combination of hydrophobic interactions, hydrogen bonding and salt bridging.[12] The specific nature of these interactions along with the relative physicochemical stability of the protein A molecule makes it perfectly suited for use as a chromatography ligand for IgG purification.

Protein A chromatography resins were initially generated using protein A produced by the *KowanI* strain of *S. aureus*.[13] With this early strain, the protein A produced was anchored to the cell wall and needed to be extracted before it could be immobilised onto the chromatographic resin. Later developed strains of *S. aureus* secreted the protein A into the culture supernatant, making recovery more straightforward.[14] Advances in recombinant DNA technology however eventually enabled expression of recombinant protein A (rProtein A) using *E.coli* as a host.[15]

Early Protein A chromatography resins were formed by immobilisation of the protein A ligands onto CNbr activated Sepharose CL 4B. This agarose derived base matrix provided a suitable support structure upon which to immobilise the selective ligand, resulting in chromatography resins which exhibited high selectivity and low non-specific adsorption of impurities. However these resins were limited in that their high compressibility placed restrictive limits upon the fluid flowrates at which chromatography processes could be operated.[12] Since then resin manufacturers have moved on to using base matrices with higher agarose compositions and cross-linking in order to increase resin rigidity and thereby enable operation under higher flowrates. Today, protein A chromatography resins are readily available from a number of manufacturers, exhibiting a wide range of properties. Manipulation of the recombinant DNA technology used to generate rProtein A ligands, in combination with advances in base matrix technology has allowed the development of resins with improved processing characteristics, such as higher binding capacities, resistance to alkali cleaning conditions and reduced ligand leakage, which as shall be elaborated on, is one of the concerns associated with the utilisation of protein A chromatography.

As stated previously, the downstream purification of the majority of mAb products, either marketed or in development, is currently based on the utilisation of process platform built around the use of Protein A affinity chromatography. Different biopharmaceutical manufacturers will have different variations of this platform, which they will apply to the purification of their various mAb products. This is not to imply that platform processes are the same as generic processes, which utilise a sequence of unit operations with a fixed set of operating parameters. Given the inherent variations in the physicochemical properties of recombinant proteins, even those of the same modality (e.g. mAbs), the use of such fully generic processes would be unlikely to yield adequate results. Instead these mAb platform processes act as a template for manufacturers when generating a purification process for a new product enabling faster process development, validation and hence speed to clinic. Whilst different manufacturers will utilise different mAb purification platforms, most of these conform to a fairly common model. Following harvest of the cell culture fermenter, the first

step in the downstream process will be cell removal through the use of centrifugation and/or microfiltration. This is then followed by a Protein A affinity chromatography step. It is the use of Protein A chromatography which is the key to enabling the use of a platform approach to the purification of mAbs.

Cell culture processes are inherently variable. No two cell lines will behave in exactly the same manner when in culture, even if measures are taken to ensure parity, such as using similar culture media, feeding strategies and operating parameters. Furthermore, this inherent variability is such that even when dealing with the culture of a single cell line, being used to generate a single mAb product, the resultant process stream, in terms of both physicochemical properties and also material composition, may still display batch to batch variabilities as high as 30%.[16] This is a problem for downstream processing, since the performance of many downstream processing operations will be dependent upon the characteristics of the feed material. For example, variations in ionic strength and pH can have a heavy impact upon the performance of ion exchange chromatography processes. Similarly variations in total protein content can impact upon the level of membrane fouling in ultrafiltration processes which can in turn affect process throughputs. Variations in total protein content will also affect the viscosity of the process stream, which will in turn impact upon the feasible operating flow rates for chromatography and membrane processes, further impacting upon process throughputs. Given the interactions between upstream and downstream, adoption of a platform process approach requires that a highly robust unit operation be utilised early on in the process train such that the process stream can be brought to a sufficiently uniform composition. This robust process would therefore essentially be acting as a "buffer" between the variable upstream operations, and the more sensitive downstream operations. The nature of Protein A affinity chromatography allows it to act as this buffer. The use of protein A affinity ligands invests the technique with a high degree of separation specificity, which is relatively unaffected by physicochemical properties such as ionic strength and pH. The former is important since the ionic strength of cell culture supernatants are typically high, whilst the latter is relevant as interactions between protein A and IgG are stable at the physiological pH at which

the majority of cell culture processes are controlled. These process characteristically, combine to endow protein A affinity chromatography with an incredibly high level of robustness, meaning that regardless (to a certain extent) of the composition of the feed material, the eluate from a Protein A chromatography process will always be of a relatively consistent nature in terms of yield and impurity profile. This enables protein A to effectively "shield" the remaining purification operations, in the process train, from variations seen upstream. This reduces the amount of process development which needs to be performed when designing a new downstream process for a new mAb product and also simplifies its validation.

Whilst Protein A chromatography does indeed display a high level of robustness and binding specificity, it is not so specific that the eluate from a Protein A process is composed only of mAbs. Impurities can also bind to Protein A resins though non-specific interactions and will elute along with the mAb. In addition to this, Protein A ligands can become leached from the resin and actually find their way into the product stream during elution. For these reasons, Protein A is not used as a single step mAb purification process. Instead a typical mAb platform process will utilise two further chromatographic operations, usually cation exchange chromatography (CEX) followed by anion exchange chromatography (AEX), in order to remove impurities such as non-specifically bound proteins, DNA fragments, mAb aggregates and also leached Protein A ligands. The combination of these three chromatography columns will normally be sufficient to generate a mAb product of sufficient purity that it may be passed on for formulation. Figure 2.3 shows the typical steps which together make up a mAb purification platform.

Harvest. Following the cell culture the first step involves removal of whole cells and cell debris from the process stream. Due to the large process volumes and relatively high cell densities which may be reached by current cell culture processes, centrifugation is the preferred over tangential flow microfiltration, for primary process stream clarification. Centrifugation offers higher throughputs than microfiltration and is also more economical to operate when dealing with large volumes, but does not provide 100% clarification. As a result the centrifugation process will normally be followed

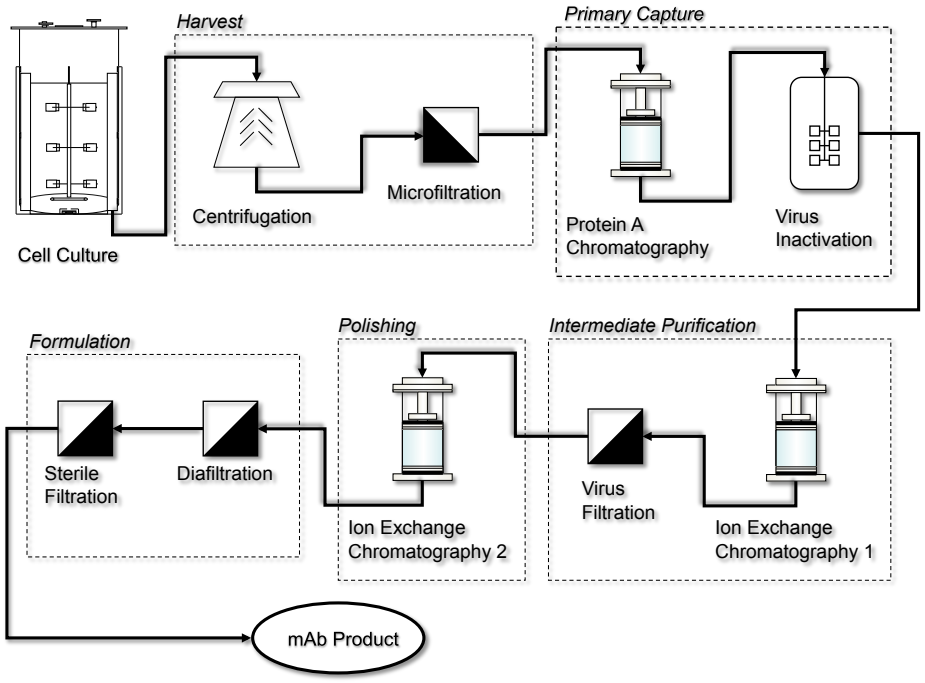


Figure 2.3: Schematic showing typical steps in a mAb platform purification process

by a depth filtration process to ensure the removal of any residual cell debris which may remain.[10]

The impurities present in the cell culture supernatant obtained following clarification of the culture broth, can be broadly classified into five major groups:[17]

- *Host cell proteins (HCP).* These are proteins which are naturally expressed by the host organism. HCP are the major contaminant present in the cell culture supernatant and can be present in concentrations of greater than 1,000,000 ppm (or alternatively 1,000,000 nanograms of HCP per milligram of mAb). The HCP levels will normally need to be reduced to below 5 ppm by the purification train.

- *DNA.* Genetic material from the host may enter the culture supernatant following breakage of whole cells during either the cell culture process or during the clarification operations. The presence of host cell DNA in the final drug product is absolutely prohibited. As a result DNA levels will usually need to be reduced to below 10ng per drug dose, from levels of possibly greater than 1,000,000 pg/mg, by the downstream process.

- *Aggregates.* These are the major product related impurity present in the process stream, and form when mAb molecules interact and become *stuck* together. The most common form of antibody aggregates are dimers, although other forms are known to occur. Aggregates can be potentially immunogenic and as a result their clearance from the final drug product is a necessity. Aggregates can make up any where between 5 and 15% of total mass of mAb present in the process stream following clarification. This will normally need to be reduced to below 0.5% by the purification train.

- *Small molecules.* The cell culture supernatant will inevitable contain many small molecules originating either from the media components used to culture the cells, or generated by the cells themselves. Clearance of small molecules is normally ascertained by determining the concentrations of several representative *marker* molecules.

- *Viruses.* Viruses may enter the cell culture broth through a number of ways. Many mammalian cell lines carry retrovirus-like particles whilst components in the culture media, particularly animal derived components such as serum if they are used, may contain viral contaminations. These viruses obviously need to be removed by the downstream purification train. Initial virus concentrations may be around 10^4 particles per millilitre. Biological pharmaceuticals are allowed to contain 1 theoretical virus particle per 10^6 doses, and so the purification process must be capable of a significant level of viral clearance.

Primary Capture. Following clarification, the cell culture supernatant is directly loaded onto the protein A affinity chromatography column in order to achieve primary capture of the product mAb. The specific nature of the affinity interactions means that during loading, only mAb should bind to the column, whilst HCP, DNA, small molecules and viruses should pass straight through. There is of course always a certain level of non-specific binding of impurities to the chromatography resin, either through hydrophobic or ion-exchange effects with the matrix support rather than the protein A ligand. These impurities may then co-elute with the product mAb, compromising

product purity. However, despite this protein A is still a high resolving operation capable of achieving purification factors upwards of 100 fold and it is during this step of the downstream process, that the majority of the purification occurs.

Along with the high purification factors, the nature of the affinity interactions between the Protein A ligand and the product mAb, and their stability under the pH neutral loading conditions means that it is also possible to achieve very high process yields using Protein A chromatography. Negligible amounts of mAb will normally be present in the flow through and wash fractions of a Protein A chromatography cycle, provided the binding capacity of the packed bed has not been exceeded. In addition, the affinity interactions between protein A and mAb may be easily disrupted by lowering the pH conditions of the system, which makes product elution fairly uncomplicated. These factors combined, allows Protein A chromatography to typically achieve yields of between 98% and 100%.[17]

Whilst Protein A chromatography does allow for high levels of purification, and yields to be achieved, its utilisation does however bring with it a few complications. Firstly, Protein A will indiscriminately bind both monomer and aggregate forms of mAb. As a result, Protein A chromatography cannot be used to remove aggregates from the process stream, something which must instead be achieved by subsequent downstream processing steps. In addition to this, and somewhat more seriously, the use of Protein A chromatography also results in the introduction of a contaminant to the process stream. With the majority of Protein A chromatography processes, small amounts of Protein A ligand can become stripped from the column during operation and end up in the antibody product pool. This *leached* Protein A can be immunogenic and cause physiological reactions and as such must be removed by subsequent downstream processing steps. The level of ligand leakage will be dependent upon a number of factors, including the resin being used, the operating conditions and the properties of the feed material. However the amount of leached Protein A in the product pool will typically need to be reduced to levels of around 10 to 12 ppm.[18]

The affinity interactions between Protein A and mAb may be disrupted by lowering the pH environment of the system. Thus elution of mAb from a Protein A

chromatography column is typically achieved using a buffer with a pH of around 3 to 3.5. As stated previously the clearance of viruses from the product pool is an absolute necessity and for biological products, regulatory authorities such as the US Food and Drug Administration (FDA) demand that the purification process should include at least two orthogonal steps for virus reduction. Virus reduction may be accomplished either through removal or via inactivation. Mechanical virus removal is fairly straightforward and may be achieved by adsorption of the product onto a chromatographic column whilst the virus is allowed to pass in the flowthrough. In this regard the Protein A chromatography step used for primary capture may be considered a virus removal step. Mechanical removal may also be accomplished using a suitably designed ultrafiltration process.

As with virus removal, virus inactivation may also be achieved via a number of methods. These methods tend to involve exposure of the virus to harsh physicochemical conditions such as acid treatment to destroy the core protein and genome of the virus, urea treatment to cause destruction of the core protein, extreme heat treatment and also exposure to ultraviolet radiation. Some of these methods are of limited use in the context of mAb purification due to the denaturing effect that they may have upon the proteinaceous product. However the exposure of the virus particles to a low pH environment is a viable option and it is one which is used by the majority of mAb purification trains. The reason for this is mainly because the low pH elution conditions from the Protein A chromatography process allows virus inactivation to be performed without the need for further treatment or adjustment of the product pool conditions. Virus inactivation can simply be achieved by holding the Protein A chromatography elution pool at room temperature for a time of between 45 and 60 minutes. It should be noted that whilst the denaturing effects of low pH exposure upon the product may be less severe than that which would be observed using, for example urea treatment, mAbs are still relatively unstable under such conditions.

As a result, following low pH virus inactivation, the elution pool will normally have to be titrated up to a pH of greater than 5, before being stored for any period of time.

Intermediate Purification. Following virus inactivation, the product pool should be comprised predominantly of the mAb product. However there will still be small amounts of HCP and DNA. The mAb contained in the product pool will also be partially made up of aggregated forms, which will need to be removed. Furthermore,the Protein A chromatography process will have introduced leached protein A ligands into the process stream, which will also require clearance. The next step in the mAb purification platform will usually be an ion exchange step, and in most cases will be a cation exchange (CEX) step, run in bind and elute mode.

In order to operate a cation exchange step in bind and elute mode, the mAb will need to be positively charged and as such loading of the product will usually be performed at a pH of between 5.0 and 6.0 (the pI of mAbs will generally be in the range of 8 to 9). Under these conditions, DNA, leached protein A and HCPs with a pI lower than the loading pH will exit the column in the flow-through. Meanwhile, both monomeric and aggregate forms of the mAb product, being positively charged, will bind to the cation exchanger. The loading pH used will need to be carefully optimised. Lowering the pH would increase the net positive charge carried by the mAb molecules, thereby increasing the capacity of the (CEX) column, however this would come at the expense of mAb protein stability. Concomitantly, higher pH values would increase the protein stability but would reduce the resin capacity.

The ionic strength of the product pool being loaded onto the CEX column must also be considered. High salt concentrations and therefore solution conductivity will *mask* the electrostatic interactions between the mAb and CEX resin and as a result inhibit product binding to the column. As a result the conductivity of the process stream to be loaded onto the CEX column should be kept low, preferably within the range of 4 to 7 mS/cm. Depending upon the conditions of the process stream, post virus inactivation, this may involve some minor adjustments such as titrating the product pool to the required pH, or in more extreme circumstances, a complete buffer exchange using ultrafiltration may be necessary. Whilst possible, the latter is unlikely given the typical conditions required for Protein A elution and virus inactivation.

Elution of mAb from a CEX chromatography column is typically achieved by

increasing the ionic strength to around 10-15 mS/cm and pH to around 7, inside the column using a suitable buffer. Increasing the pH will reduce the net positive charge carried by the mAb molecules thereby weakening the interactions between the product and the CEX resin, whilst increasing the ionic strength will serve to further disrupt the electrostatic attraction between the two, allowing product elution.

As stated previously, both monomeric and aggregated forms of mAb will both bind to a CEX column. However the strength of these interactions are slightly different, with aggregates generally having a higher electrostatic affinity to the CEX ligands than the monomer. Figure 2.4 shows a typical elution chromatogram which would be obtained for a mAb product from a CEX column. Also shown is the typical monomer percentage of the column output over the course of the elution. Because of the higher strength of the electrostatic interactions with the CEX ligands, aggregates will tend to be more difficult to elute from the column and as a result will emerge in the *tail* end of the elution peak. This behaviour means that it is possible to achieve a significant level of aggregate clearance by simply collecting only the front bulk of the elution peak and not the tail, as shown by the dashed line in Figure 2.4

As with the majority of downstream purification processes, a trade-off will typically need to be made between the product purity, in terms of monomer composition, and also the process yield. Cutting off a large portion of the elution tail will ensure a high level of product purity, however this will come at the expense of a reduction in process yield. The magnitude of this trade-off may be alleviated to a certain extent by increasing the resolution between monomeric and aggregate forms of the mAb, for example through the use of a gradient elution in which the pH and ionic strength of the elution buffer is gradually increased. Such a strategy would cause a spreading of the elution peak, allowing the high aggregate containing tail to be cut with a reduced impact upon process yield, however this would obviously impact upon operational complexity as well as cause a dilution of the product pool.

Elution in CEX chromatography processes may often be carried out using a gradient in order to boost the resolution of components. However since in the case of the mAb platform process, the CEX step is being used following a Protein A chromatog-

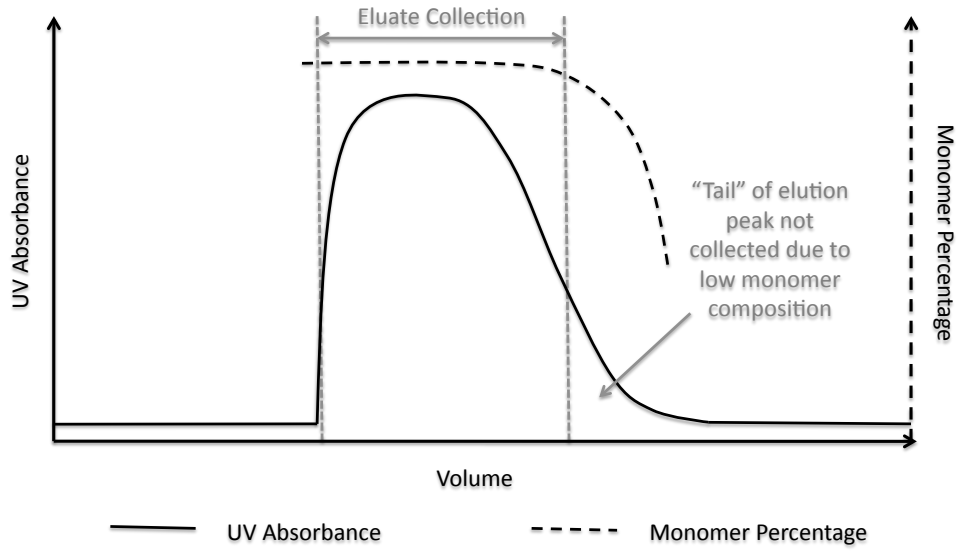


Figure 2.4: Schematic showing a typical CEX chromatogram for a mAb product

raphy step, the relatively high purity of the feed makes the use of a gradient elution unnecessary and a step elution will usually be sufficient.

Following CEX, the next step of the intermediate purification process will typically be a virus filtration. Discounting the two chromatography processes (Protein A and CEX) which would, to this stage have been utilised, this virus filtration step serves as the second of the two prerequisite virus reduction step needed in any process generating a biological therapeutic product. Virus filtration can be accomplished using either depth filters with pore sizes in the range of 20 to 40nm, run in dead end mode, or by using tangential flow ultrafiltration with membrane cut-offs of less that 300 kDa .[19]

Polishing and Formulation. Following CEX chromatography and viral filtration, a final selective chromatography step will usually be used to remove trace impurities from the product stream. This final step will usually be an Anion Exchange (AEX) Chromatography operation run in flow through mode. Operating at a pH value of between 0.5 to 1 units below the pI of the mAb, thereby ensuring the molecules carry

a positive charge, the products can be made to flow through the column. Furthermore most HCP will have a pI lower than that of the mAb and as a result, under such pH conditions will bind to the positively charged resin. Endotoxin and DNA will also strongly bind to the AEX column under these conditions. Again conditioning of the product pool following the intermediate purification steps may be necessary in order to reach the required pH and solution conductivity conditions, and under some circumstances, a complete buffer exchange using an ultrafiltration step run in diafiltration mode may be necessary. Following this final AEX step, the purity requirements of the mAb product should have been reached. The only steps remaining are a diafiltration step to allow firstly suspension of the mAb in the desired formulation buffer, and also concentration of the mAb to levels required for delivery to patients. This would then be followed by a final sterile filtration step before product filling. Figure 2.5 shows a breakdown of the steps of a typical mAb platform purification process and the impurities removed by each unit operation.

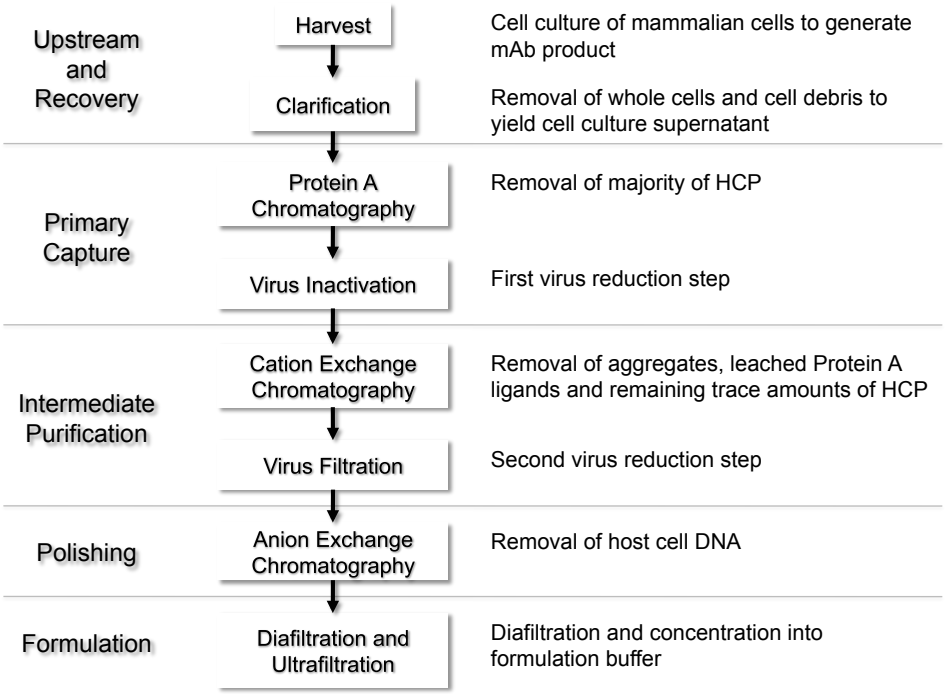


Figure 2.5: Breakdown of a typical mAb platform purification process and the impurities and contaminants eliminated by each step in the process train

As stated previously, different manufacturers will each have their own mAb pu-

rification platform process, however each of these platforms will essentially be a variation upon the Protein A - CEX - AEX three column train. Some will have a hydrophobic interaction chromatography (HIC) step instead of the final AEX column. Other platforms may utilise this HIC step in addition to the standard three column train, or instead of an additional HIC step, may employ an additional size exclusion chromatography (SEC) column. Table 2.1 shows the purification trains used to purify 6 different marketed mAb drugs; Herceptin™, Rituxan™, MabCampath™, Synagis™, Remicade™ and Simulect™.[19]

Table 2.1: Purification process trains for marketed mAb products
(Data taken from Sommerfeld et al., 2005[19])

	Herceptin™	Rituxan™	MabCampath™	Synagis™	Remicade™	Simulect™
Clarification	1	1	1	1	1	1
Protein A Chromatography	2	2	2		2	2
Virus Inactivation	3	3	3	4	3	3
Cation Exchange	4	5	4	3	4	5
Anion Exchange	5	4		2, 6	6, 7	4
Hydrophobic Interaction	6					
Size Exclusion			5	8		
Virus Clearance		6	6	5, 7	5	6
Sterile Filtration	7	7	7	9	8	7

As can be seen there are definite similarities across all of these mAb purification processes. All processes (with the exception of that used to purify Synagis™) follow clarification with a Protein A affinity chromatography step for primary capture of the mAb product. This is then followed by either one or more ion exchange chromatography steps. If the purity requirements are not met by these steps, then additional chromatography processes will be used, such as hydrophobic interaction chromatography and size exclusion chromatography.[19] In addition to the process shown in Table 2.1 development work has also been done toward creating a mAb purification platform where the CEX column is removed from the standard Protein A - CEX - AEX platform in order to form a two column (Protein A - AEX) process, whilst research has also shown that it is possible to have a two column mAb purification process in which the CEX and AEX columns are replaced by a multi-modal anion exchanger (Capto adhereTM) chromatography column.[9, 20] Regardless, the one thing almost all

mAb platform processes have in common, is the utilisation of a Protein A affinity chromatography column at the start of the downstream processing train in order to achieve primary capture of the mAb product, as this is the key to being able to employ the platform approach. It is this heavy dependency upon the use of Protein A for mAb purification which has been the cause for some concern amongst process engineers involved in the purification of mAb products.

2.1.3 The Constraints of Packed Bed Chromatography

The high dependence upon the use of Protein A chromatography for the purification of monoclonal antibody products has given rise to concerns amongst the biomanufacturing community regarding the potential constraints which may be encountered as a consequence of the technological advance seen in upstream operations. These concerns may be broadly grouped into two categories. Firstly there are the issues associated with the cost of chromatography, and secondly there are those related to the potential manufacturing capacity bottleneck which chromatography may represent.

The Cost of Chromatography

The cost of goods (CoGs) can be defined as the investment required to manufacture a unit mass of product. As a result, the CoGs will typically have units of \$/g or £/g which will account for the cost of all the raw materials and consumables required to produce a single gram of product. Biopharmaceuticals are currently considered to be low volume, high value products and as such the CoGs associated with biomolecules are typically and justifiably larger than that of their small molecule counterparts. However biomanufacturers have recently come under pressure to reduce the CoGs associated with their products. As a result of current global economic difficulties, healthcare providers with limited resources at their disposal, are beginning to use cost/ benefit models to evaluate the therapies which are most worth procuring, and in extreme cases denying patients access to medications which are too expensive and are deemed to not provide a sufficient improvement in quality of life, such was the case with Avastin™ in the UK, in which bowel cancer sufferers were denied access on

the NHS due to the drug being assessed as not being cost effective. As a result of this increased pressure to reduce process costs, certain unit operations within downstream purification trains have recently come under increased scrutiny.

Protein A affinity chromatography processes, as is indeed the case with all chromatography processes, are scaled based upon the mass of product which needs to be captured rather than the volume of process material. A 10,000L fermenter may be used to culture a cell line expressing mAb at a concentration of 1g/L, thereby producing a total of 10kg of mAb per batch. If the same fermenter is used to culture a cell line expressing mAb at a concentration of 10g/L, the volume of the cell culture will still be 10,000L, but the mass of mAb contained within will now 100kg. Thus the Protein A chromatography column will need to be either made 10 times as large as that which was used to capture the 10kg batch or instead run for a greater number of cycles per batch.

The requirement for larger columns is a concern primarily because of the associated costs. The protein A ligands used in the majority of affinity resins is a recombinant protein, manufactured using validated fermentation and downstream processes.[21] Since the ligand itself is a biological product, the production of Protein A chromatography media is expensive, particularly compared to other chromatographic resins (e.g. IEX, HIC, SEC), and as a consequence the economies of scale associated with its procurement are almost non-existent. Protein A chromatography resins will typically cost around $10,000 per litre, which is over three times as expensive as most ion exchange resins. With most chromatography media suppliers, this is a figure which does not vary appreciably with the volume of media being purchased.

The consequence of this is that whilst increasing cell culture titres make upstream operations cheaper in terms of specific production costs (e.g. $ per gram of mAb), the same does not apply to Protein A chromatography. Figure 2.6 shows how the cell culture mAb titre affects the relative cost contributions of both upstream and downstream processing costs.[1]. From this it can be seen that at low product concentrations upstream operations contribute a significantly larger proportion of the production costs than downstream processes. However as soon a the product titre

rises above 1g/L, downstream processing costs start to predominate. At a cell culture mAb titre of 2 g/L, downstream processing makes up over 80% of the total production costs. Unsurprisingly, of all the unit operations in the mAb purification platform, chromatography processes are the biggest cost contributor and in particular Protein A affinity chromatography, to which almost half of the overall downstream process costs may be attributed, as shown in Figure 2.7.

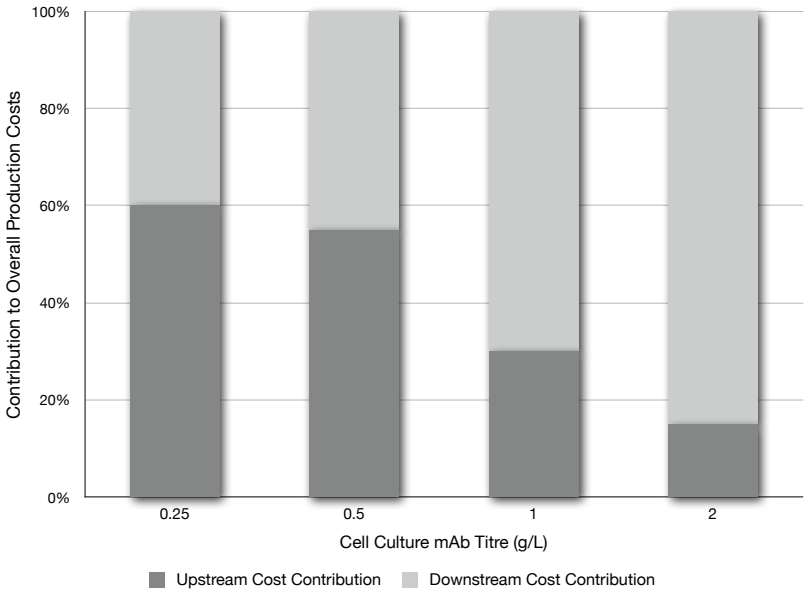


Figure 2.6: Chart showing percentage contribution of upstream and downstream operations to the overall production cost of a mAb drug

The disproportionate cost contribution made by downstream processing is only further exacerbated by the advances observed in upstream operations over the past decade, and in particular those seen over the past several years. So whilst upstream operations become increasingly more economical, the cost of downstream processing remains relatively unchanged, something which is particularly troubling, in light of the increasing pressure from healthcare providers on biopharmaceutical manufacturers to reduce the costs of their drugs.

The scale of the costs which might be associated with a Protein A affinity chromatography process, used to capture mAb from a high titre cell culture supernant may be illustrated with a simple example, in which a 10,000 L fermenter is used to

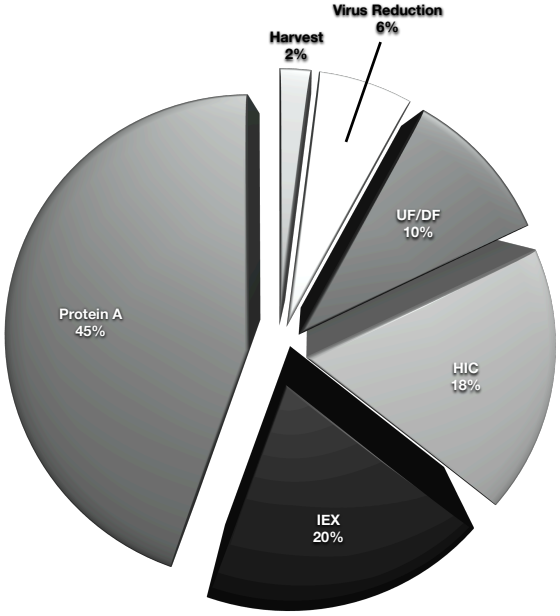


Figure 2.7: Breakdown of major cost contributions to downstream processing by unit operation

culture a mammalian cell line expressing mAb at a titre of 10g/L. This cell culture would be capable of generating 100 kg of mAb per batch which would require a 2,000L Protein A column, in order to capture the product in a single pass (assuming an appropriate mobile phase flow rate is used in order to obtain a dynamic binding capacity of 50g/L). At a price of $10,000 per litre, this 2,000 Litres of resin would cost $20 Million. Considering that facilities will generally have at least an additional column in inventory in case of any failures, the total Protein A present at this particular facility will have a total cost of $40 Million. This is a sizable investment, particularly when placed into perspective. The capital cost of a bioreactor along with the control skids added to the capital cost of the chromatography column itself, the associated control skid and a packing skid would only amount to just under $3 Million.[9]

Whilst large, the costs of Protein A are of course not completely unjustified. In addition to the high performance and robustness offered by the technique, as detailed previously, it should also be noted that the relatively long lifetime of current resins means that the incurred costs may be spread over a large number of manufacturing cycles, such that the actual cost of Protein A resin on a per cycle basis is actually $50

per L per cycle. Thus the $20 Million column described previously would only have a cost of $100,000 per cycle, meaning that the specific cost of Protein A resin would only be $1 per gram of mAb captured. From this perspective, the cost of Protein A is not a significant issue. However the calculation is based on the assumption that the resin will indeed be used for 200 cycles, when there are in fact a number of scenarios in which such a high number of reuses will never be reached. For example this is not the case during clinical production campaigns where the same resin may only be used for 1 - 20 cycles, to generate a small amount of material for clinical trials after which it must be discarded .[9] Even a column being used to produce material for commercial supply may not be re-used for the full lifetime of 200 cycles. As shall be elaborated on later in this chapter, one industry trend currently occurring is the use of multi-product facilities operating short intensive manufacturing campaigns, producing a wide range of different products. In such cases a Protein A column may only be used for a fraction of its possible life time before it is disposed of as the facility is switched over to a new product. In addition to this, the risk of contaminations and failures during operation can also not be discounted and such eventualities may occur well before a resin has reached the end of its lifetime. Add in to this the fact that the 200 cycle lifetime is provided as a rough guide and that actual resin lifetimes may vary significantly depending upon a wide range of different factors such as the properties of the feed and the operating conditions used, it can be seen that the re-use of resin does not necessarily eliminate the cost issues associated with the use of Protein A chromatography.

The Potential Capacity Bottleneck

The cost of Protein A is a tangible property and therefore the economic issues surrounding its usage may be more immediately obvious and compelling. However somewhat more profound and equally relevant are effects that the capacity constraints of not only Protein A, but all packed bed chromatography operations, have upon the productivity of biomanufacturing processes.

The binding capacity of a chromatography resin describes the mass of product

which may be captured per unit volume of packed bed, and may be expressed in one of two ways. The equilibrium capacity of a chromatography resin, is a measure of the capacity the resin would exhibit if it were placed in a static batch system. For example this would be a measure of the amount of antibody which could be bound to a given volume of Protein A resin were it to be placed into vessel containing an excess volume of a highly concentrated pure mAb solution, for an infinite period of time. The equilibrium capacity of a chromatography resin will be determined by the concentration of ligands immobilised onto the matrix support. High ligand concentrations will generally result in higher binding capacities as there will be a larger number of binding sites available per unit volume of bed, assuming of course that such sites are accessible to the target molecule.

Since a chromatography process is not a static system, and the feeds used are generally multi-component complex mixtures, the actual capacity of a chromatography column will never be as high as the equilibrium capacity. Instead, a more useful measurement is the Dynamic Binding Capacity (DBC), which may be thought of as the capacity exhibited by a packed bed under operating conditions. Whilst for a given chromatographic resin, the equilibrium capacity will remain fairly constant (assuming that the concentration of the target molecule is maintained), the DBC is a much more variable quantity, dependent upon a number of process specific characteristics, of which principal amongst them is the mobile phase flow rate. In order for a target molecule inside a chromatography column, to move from the bulk fluid to the binding site of a ligand, it must go through a number of mass transfer processes as shown in Figure 2.8. Firstly there are the bulk convection forces taking the molecule from the bulk fluid to the resin particle. Once there the particle diffuses through the stagnant film at the particle surface before diffusing into the pores of the particle, where the ligands are immobilised. It is only then that the binding kinetics take over and the molecule is captured. In order for a molecule then to bind to a packed bed, it must be given sufficient time to go through all of these mass transfer processes. The total time a single molecule spends inside a packed bed is known as the residence time. In the previously described batch system used to measure the equilibrium capacity, the

residence time is infinite and as a result molecules have an unlimited amount of time to reach a binding site. Since the system is left to reach equilibrium, it can be ensure that each and every one of the available ligands present in the system is used and that the full capacity of the resin is being utilised.

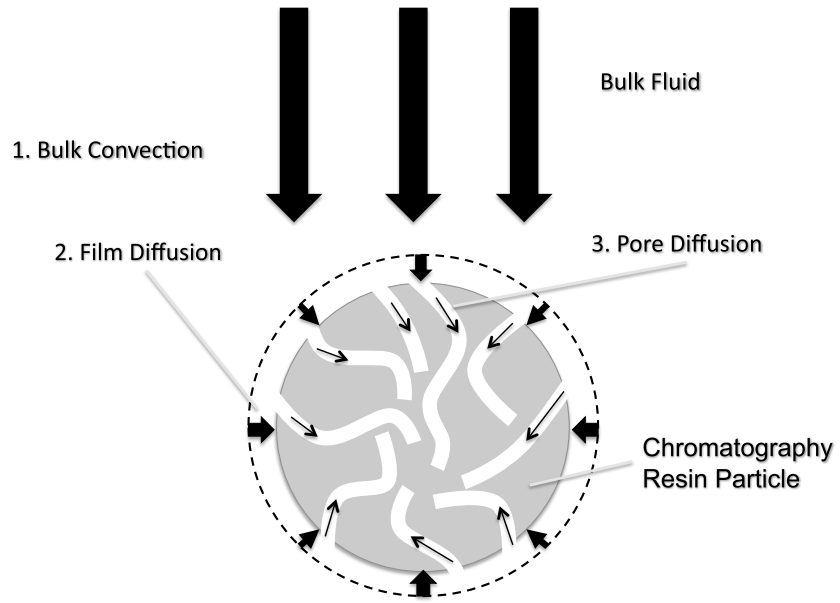


Figure 2.8: Schematic showing the mass transfer processes which a solute molecule must undergo before it may bind to an immobilised ligand during packed bed chromatography

This however is not necessarily the case when dealing with an actual chromatography process, wherein the residence time is determined by the flow rate of the mobile phase through the column. As the mobile phase flow rate is increased, the residence time decreases and there is less time available for molecules to go through all of the necessary mass transfer processes in order to reach and bind to the ligands inside the pores, and instead simply move straight through the column. As a result whilst the ligands on the particle surface and those close to the entrances of pores may all bind to a product molecule, those further inside the pores do not, as there is insufficient time available for solute molecules to move this deep into the resin particles. It is due to these *unused* ligands that the dynamic binding capacity of the bed is observed

to be lower than the equilibrium value. The equilibrium capacity may therefore be thought of as a theoretical maximum, and the role of the process engineer is to design their chromatography process in order to come as close to this maximum as possible without compromising other process characteristics such as throughput and productivity.

The issue now with Protein A chromatography is that a limit has been reached with regards to the equilibrium capacities which may be obtained. With the structure of current base matrices, methods of immobilisation and the rProtein A ligands being used, it is unlikely that the concentration of ligands can be increased past the levels displayed by the current generation of resins. Accounting for the mass transfer limitations associated chromatography processes under actual operating conditions and it may be argued that it will not be thermodynamically possible to significantly increase the *dynamic* binding capacities of Protein A resins beyond 50g/L. Indeed, most modern Protein A chromatography processes are operated with a DBC of between only 30 and 40g/L. This constraint on capacity obviously has implications upon the cost of a Protein A chromatography process, particularly when dealing with increases in cell culture titre as larger columns will be required to capture all of the generated product. However increases in column size will also have implications upon the productivity of the chromatography process. These productivity constraints can be attributed to a property of all chromatography columns, known as the critical velocity (u_{crit}).

During a chromatography process, the mobile phase will exert a drag force upon the resin particles as it moves through the column. This drag force will serve to pull the resin particles down and in effect compress the packed bed. Compression of the packed bed reduces its porosity (ϵ) which in turn increases the pressure drop across the column (ΔP). If the mobile phase flow rate is incrementally increased, further compression of the column will cause the porosity of the bed to continue to decrease, whilst (ΔP) across the column will rise. If this increasing of the mobile phase flow rate is continued, then a point will be reached whereby the porosity of the bed reaches a minimum and (ΔP) rises to infinite without any further increase in mobile phase velocity. The superficial fluid velocity of the mobile phase at which

this occurs is known as the critical velocity. The u_{crit} of a chromatography column therefore describes the maximum flow rate at which the mobile phase may be delivered to the packed bed and it places a practical constraint upon the feasible flow rates at which a chromatography process may be operated, which in turn provides a limit for the productivity of a given chromatography column.

The u_{crit} of a packed bed can vary and is dependent upon a number of different factors, including the compressibility or "softness" of the resin and the viscosity of the feed material. However the factor which has the greatest influence upon the u_{crit} of a packed bed, is its dimensions. Taller columns will tend to have lower u_{crit} values than shorter ones due to the added compression effect upon resin particles located in the lower regions of the bed, exerted by the weight of the particles above it. Wider columns will also have lower u_{crit} values than narrower ones due to the loss of frictional support forces supplied by the walls of the column housing the packed bed. A number of studies have been performed, aimed at developing predictive models which enable the critical velocity of a packed bed to be determined using data obtained from small scale experiments.[22, 23]. These predictive models vary in complexity and the degree of ease at which they might be used. One relatively straightforward correlation which has been proposed, relates the u_crit of a chromatography column to it's initial bed height (L_0) and the column diameter, through the use of system specific empirical constants.[22]

$$u_{crit} L_0 = m\left(\frac{L_0}{D}\right) + b \tag{2.1}$$

The empirical constants m and b shown in Equation 2.1 are dependent on system specific parameters such as the rigidity of the resin and the viscosity of the feed material. In order to use this correlation, small lab scale experiments would first need to be done in order to determine the values of m and b. Once these are obtained, Equation 2.1 may be used to determine the critical velocity of any sized column, assuming the aforementioned system specific parameters from the small scale experiments are maintained.

The impact of binding capacity and flow rate constraints upon the productivity of a chromatography process can be illustrated using a relatively simple example in which a Protein A chromatography column is scaled up in order to handle an increase in feed titre. This example is summarised in Figure 2.9. In the base case a Protein A column is being used to process feed from a 20,000L cell culture process, with a mAb titre of 1g/L. Based on an assumed DBC of 40g/L, in order to process all of this material in a single cycle, a 500L Protein A column would be required. To achieve this volume, a 200cm diameter column is packed with Protein A resin to a bed height of approximately 16cm. Using Equation 2.1, the critical velocity of this column may be calculated to be approximately 320 cm/h. The empirical constants m and b were assumed to be 1058 cm^2/hr and 5055 cm^2/hr respectively. These values were obtained from literature and may be assumed to be close to those which might be observed for a typical Protein A process.[22]. The buffer volumes required to complete each step of Protein A chromatography cycle are shown in Figure 2.9 and are typical of those which might commonly be associated with this type of process. If this chromatography process were to be operated at a fluid flow rate equal to the critical velocity, the total process time would be just over 3 hours, and the productivity would be calculated to be over 6000 g/hr. This is obviously a fairly optimistic calculation as it is unlikely that a column would ever be operated close to the critical velocity, and indeed operation *at* the critical velocity would be technically impossible. In addition to this, the accuracy of the correlation shown in Equation 2.1 at extreme aspect ratios (L_0/D) is somewhat questionable. However assuming operation at the critical velocity in this manner does allow the calculation of a theoretical maximum for the process productivity.

As stated previously, if the titre from a cell culture process increases by 10 fold, and assuming the culture volume is maintained, the Protein A column used to capture the mAb would also need to increase in volume by ten fold. This can be accomplished by simply increasing the size of the chromatography column. However taking such an approach does have potential consequences. Firstly there are constraints upon the size of chromatography column which may be used. Currently the largest commercially available column has a diameter of 2 metres. Larger columns than this are not

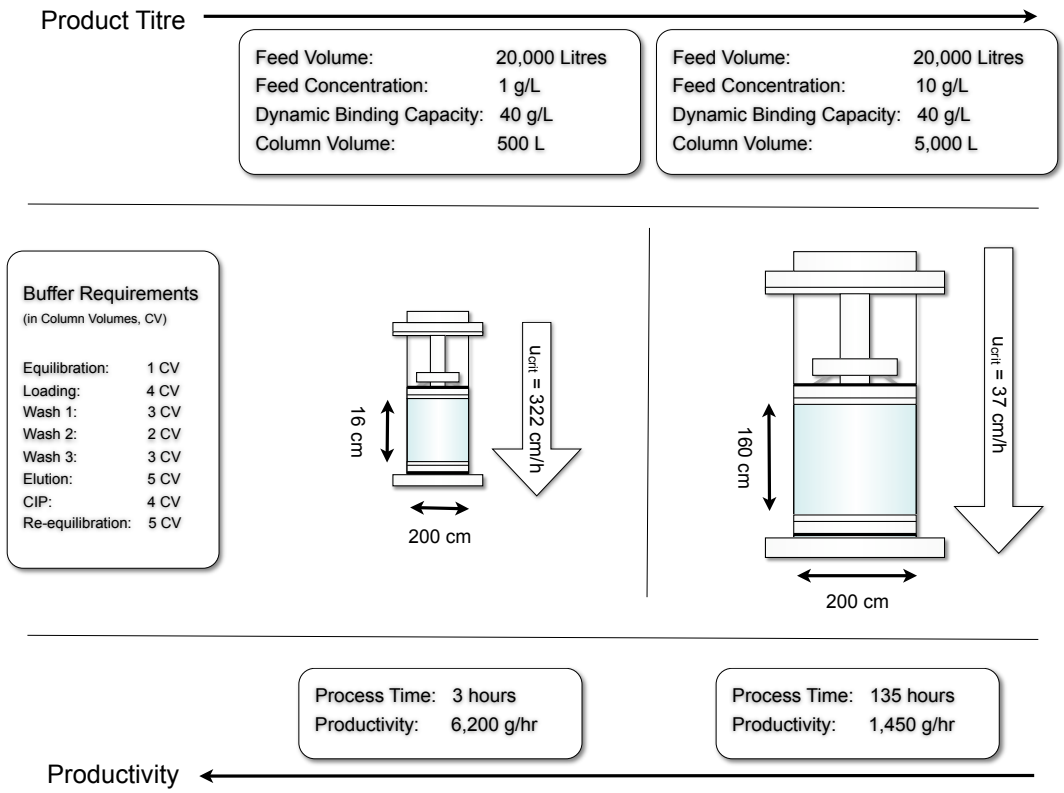


Figure 2.9: Comparison showing the relationship between the productivity of a Protein A chromatography process being used for primary capture of a mAb product, and the cell culture titre, accounting for column compression and flow velocity constraints

available due to difficulties with ensuring uniform fluid flow across the entire bed. The scale up of chromatography processes is typically achieved by increasing the column diameter whilst maintaining the bed height in order to maintain the superficial fluid velocity, ensuring that it does not exceed u_{crit}, and that the residence time and dynamic binding capacity is maintained. If the required bed volume is such that a column of greater than 2 metres in diameters is required, the only recourse is then to increase the bed height. However, in order to maintain the residence time and also importantly, the productivity of the process, the superficial fluid velocity must also be increased in response to this change in bed height. The height and critical velocity of a packed bed, as previously detailed, share an inverse relationship. As bed height increase, the critical velocity decreases. A scenario can therefore be envisaged, whereby the required bed height is so large that the resultant required superficial fluid velocity exceeds the constraints of u_{crit}. In such cases the fluid flow through the column must be decreased, resulting in an increase in processing time and a decrease in the productivity of the chromatography process.

An alternative strategy would be to employ multiple shorter beds, which are then run in parallel. Employing such an approach would effectively eliminate any concerns of productivity limitations since the throughput of the Protein A capture step may be increased by simply increasing the number of columns used in parallel. Doing so however, brings up an important consideration regarding plant space. Increasing the total volume of the Protein A step, whether it be by using one large column, or multiple smaller ones, will increase the floor space requirements within a manufacturing facility, since not only must the plant accommodate larger chromatography columns, but also larger buffer tanks, in process storage tanks, solution preparation tanks etc. This becomes an even greater problem when the idea is to retro-fit an existing plant to handle a new high mass process, since it simply may not be physically possible to reconcile the increased space requirements in the current facility.

If the idea is indeed to utilise an existing facility to house a new high mass process, the need for plant rebuilding may be avoided by simply operating the smaller Protein A column already present in the plant over a larger number of cycles, assuming

the issues associated with the need for larger buffer volumes may be overcome using concentration solutions and in line dilutions and that the need for larger in-process storage tanks may be accommodated. However operating a chromatography column for a greater number of process cycles has the implication of increasing the overall process time and as a result, the overall productivity of the chromatography process will again become constrained. It can be seen therefore why Protein A chromatography, and all packed bed chromatography processes for that matter, may be considered a potential productivity bottleneck when it comes to dealing with high mass processes, particularly when it comes to retro-fitting an existing facility in order to accommodate a new high titre process. However these issues of productivity limitations are not eliminated even if a plant is to be built from scratch, since the engineer designing the plant will always have to keep in mind the potential need for ever larger downstream processing capacities.

2.2 Future Challenges for Downstream Purification

2.2.1 The Multi-ton Manufacturing Process

It could be argued that the cost issues and productivity constraints associated with the use of packed bed chromatography are not insurmountable, particularly from the standpoint of commercial manufacturing. As described previously, the long cycle lifetimes of current Protein A resins means that their costs may be spread over the duration of a manufacturing campaign, making the specific costs relatively low at approximately $1 per gram product. Similarly, through appropriate facility design and optimisation of packed chromatography processes, it should be possible to develop downstream purification processes with productivities in the multi-ton range. Indeed, in a previous study, the argument was put forward that despite the perceived capacity limitations associated with the current mAb manufacturing platform, built around the use of packed bed chromatography, it should be theoretically possible to develop a

manufacturing facility with a production rate of up to 10,000kg of mAb a year.[9]

Several conclusions were made from this study. Firstly it was surmised that with regards to dealing with commercial manufacturing, the cost of chromatography, and in particular Protein A chromatography, is not a significant issue. The downstream process housed in this hypothetical facility utilised a 1,500L Protein A column, and it was assumed that a dynamic binding capacity of 50g/L could be achieved, with a resin lifetime of 200 cycles. Based on these parameters, the specific cost of Protein A chromatography was calculated to be close to $1 per gram of product. The only major cost related issue was the high upfront capital investment in Protein A resin required. The 1,500L of Protein A resin would currently cost approximately $15 million. Assuming that a back-up column would also be required in inventory, the total cost of Protein A resin held in the facility at any one time would amount to a significant figure of $30 million. However this cost, assuming that the resin can indeed be used for 200 cycles and no failures are encountered, can be distributed over the annual production campaign, resulting in the relatively low specific production cost associated with Protein A quoted. It was also concluded that with a throughput of 10,000kg mAb per year, the capacity constraints imposed by packed bed chromatography could also not be considered an issue.

The study provides a compelling counter-argument to the perceived issues associated with the current mAb manufacturing platform. However it should be noted that the conclusions drawn are based on a fairly idealised scenario in terms of the performance of the unit operations and the also the circumstances of the biomanufacturer, and as a result come with a number of key caveats.

The first factor which must be taken into account is the purification performance of the process train, and specifically that of the anion exchange chromatography step. In the study the downstream purification was based upon the utilisation of a two column process. The first is the Protein A column for product capture whilst the second is a anion exchange (AEX) column run in flow-through mode. The study is based upon the assumption that this two step process is sufficient to provide the necessary level of impurity clearance. Cation exchange (CEX) has been more traditionally used as the

second, intermediate purification step in the mAb purification platform, as it allows for a high level of impurity clearance. The disadvantage of using CEX chromatography however is that it must be run in bind and elute mode, due to the high pI of most mAbs. AEX chromatography is normally utilised as a final polishing step and is run in flow-through mode. As a result the processing time associated with the AEX is generally shorter than that associated with chromatography processes run in bind and elute mode. By eliminating CEX and utilising AEX as both an intermediate purification and also polishing step, the productivity of the downstream process may be dramatically increased, explaining the utilisation of this particular strategy for this study.

Such an approach however may present some issues. It could be argued that the use of a single step for intermediate purification and polishing, might not provide sufficient impurity clearance. With AEX, run in flow-through mode, conditions are chosen such that impurities bind to the positively charged resin, whilst the product mAb passes straight through. Operation in this mode may not allow impurities with comparable charge characteristics to the product to be removed. In CEX, careful selection of wash strategies and elution peak cutting criteria may allow for better impurity clearance, however operation in flow through mode makes this more difficult.

To address this, it was proposed that the AEX column be operated in a mode known as Weak Partitioning Chromatography (WPC). A variation on the standard flow-through mode of operation, in WPC, conditions are chosen such that as well as impurities, a significant amount of product also binds to the positively charged chromatography resin.[24] Under such stringent binding conditions, higher levels of impurity clearance have been observed. Whilst such a strategy may allow for sufficient removal of impurities such as HCP and DNA, the removal of product related impurities, specifically mAb aggregates may be more problematic. As has been described, CEX can be effectively used for the clearance of aggregates, as they generally have higher affinities for the negatively charged CEX resin. Clearance of aggregates using AEX, even in WPC mode may not be sufficient for products with high levels of product related impurities. The study quotes high levels of removal of all impurities,

however the robustness of such performance over a range of products cannot be determined. However the assumption is that the two step process is enough to achieve the desired levels of product purity required for a bulk drug substance.

The second assumption made is with regards to the capacity of the Protein A chromatography column. In the study, the productivity of 10,000 kg per year was based on the assumption that a dynamic binding capacity of 50g mAb/ Litre Resin may be achieved on Protein A. Such capacities are not currently commonly achievable. Indeed the study stated that the figure of 50g/ L resin is based upon the extrapolation of what should be possible based on existing data, rather than a reflection of what had actually been achieved. Furthermore, dynamic binding capacities will vary from product to product and as a result whilst it might be possible to load the Protein A column with product A to 50g/L Resin, it might not necessarily be the case for product B.

Achieving the required impurity clearance on AEX and also obtaining a Protein A dynamic binding capacity of 50g/ L resin are both process performance related factors, and it could be argued that through effective process development, it might be possible to reach these levels. The conclusions from the study do however come with one final caveat, and it is a significant one.

The ability to manufacture 10,000kg of product annually is based on the assumption that a facility is purpose built in order to house this high productivity process. Such an option, whilst not unrealistic, is possibly not one which would be readily available to the majority of biomanufacturers. Instead, a more likely alternative would be to retrofit an existing manufacturing facility to house the 10,000kg process. However taking such an option reveals the productivity constraints imposed by downstream purification and in particularly packed bed chromatography. In the study the process was designed to process 15,000L of clarified fermenter harvest with a product titre of 5g/L. If this process were to be retrofitted into a plant with the same fermentation capacity (i.e. 15,000L), but with a downstream purification train designed to only handle a product titre of only 1g/L, then the downstream purification unit operations which scale based upon the mass of product to be process, along with all the

associated ancillary equipment such as buffer and storage tanks, would all need to be scaled-up by a factor of 5-fold. If insufficient space is available to accommodate this increase in process scale, then significant capital investment would be required for facility expansion.

As a result, whilst the hypothetical 10-Ton mAb process shows that it is possible to use a variation of the current mAb purification platform to produce multi-ton amounts of product, it does not address the fact that downstream processing and particularly packed bed chromatography, which scales with the mass of material which needs to be purified, places a very definite constraint upon the maximum productivity of the plant. The case study simply showed that it is possible to shift the constraint by increasing the size of the facility. Thus whilst a custom built 10-Ton manufacturing facility may be capable of producing 10 Tons of mAb a year, in all likelihood it would not be able to manufacture 11-tons.

2.2.2 Flexible Multi-Product Facilities

Whilst it is evident that packed bed chromatography does indeed place a definite constraint upon productivity, the concept of downstream processing presenting a bottleneck to manufacturing is based on the assumption that commercial demand is actually driving productivity requirements beyond these constraints. Thus whilst it may be necessary to invest vast sums of capital in order to increase the production capabilities of a facility to enable it to manufacture 10 tons of mAb per year, are such levels of productivity actually required?

A mAb therapeutic may be assumed to have a dosage requirement of 2-5g per patient per year.[19] If it is assumed that this product has a large potential market of approximately 250,000 patients, then this would amount to an annual product demand of 1.25 Tons of mAb per year. Current mAb processes manage productivities of 5-10kg per batch (assuming a cell culture titre of approximately 1g/L and a culture volume of 10,000-15,000 L). Assuming that a manufacturing plant can operate 140 batches per year, this would give the plant a manufacturing capacity of up to 1.4 Tons mAb per year, which exceeds the market demand. Based on this it could be argued

that whilst the use of packed bed chromatography may indeed constrain the productivity of a manufacturing facility, current market demands are not sufficient to test these constraints. Indeed an alternative argument has been put forward suggesting that the the future challenge for biomanufacturing is not in developing facilities capable of producing multi-ton amounts of product, but moreover, flexible multi-product facilities capable of producing smaller quantities of a wide range of different products.

A major factor influencing such a trend is the diminished likelihood of developing so-called "blockbuster" biotherapeutics. It could be argued that most of the major medical indications, such as rheumatoid arthritis, anaemia and diabetes, are already being served by a number of current protein drugs. As a result, drug developers are having to develop drugs for indications with relatively smaller associated patient populations. Furthermore, the level of competition in the biopharmaceutical industry has also increased significantly over the past decade, meaning that the market share which may be achievable by a single drug is also markedly diminished. These factors combined means that the annual production requirements for a new mAb product will unlikely breach the 1,000kg mark.[25]. Indeed of the currently marketed MAb products, aside from blockbusters such as Rituxan and Avastin, most have annual production scales ranging only between 50 and 100kg as shown in Figure 2.10

These required annual production volumes call into question the need for high capacity production facilities, such as the one described previously in Section 2.2.1. For example whilst a 15,000L fermenter, housing a cell culture process capable of expressing mAb to a titre of 5g/L, would be able to produce over 10-Tons of mAb per year, the same upstream process would actually be capable of producing the annual production requirement for a "typical" mAb product in a single batch. Thus it could be argued that the future of biopharmaceutical manufacturing lies in a reduction, rather than increase, in production scales.

With the reduced likelihood of developing a blockbuster mAb drug, biopharmaceutical developers will most likely look instead towards developing and marketing a wide range of antibody drugs, targeting a number of different indications. As a result, rather than generating multi-million dollar annual revenues off a single blockbuster

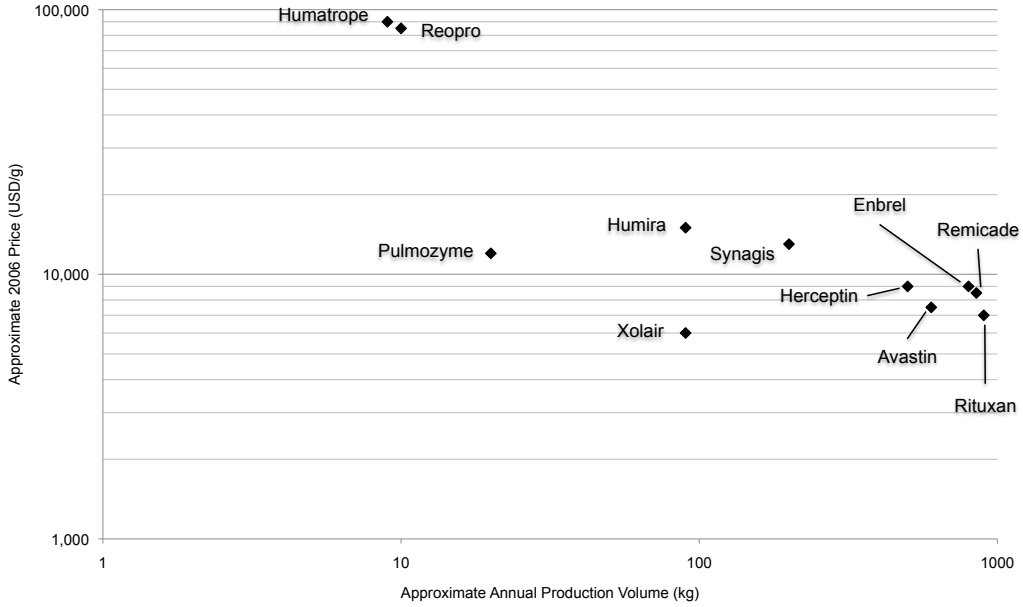


Figure 2.10: Chart showing the annual production volume and pharmacy price of marketed mAb drugs (including mAb based fusion proteins) in 2006

Data taken from Jagschies, 2008 [25]

drug, these companies will try to gain the same revenue, spread across a number of different products. The challenge facing these companies will be to find ways of manufacturing their diverse portfolio whilst controlling both capital expenditure and production costs.

One available option would be to utilise existing manufacturing facilities to produce multiple products. Increases in upstream productivity make such an option feasible since, as described previously, a facility housing a 15,000L fermenter should be capable of generating sufficient material to supplying the necessary demand for a new mAb product in a single batch, assuming that a cell culture titre of 5g/L or greater can be achieved. Multiple manufacturing campaigns through the same facility would then allow the demands for a number of different products to be met, without the need for any additional capital expenditure for plant development and building.

The multi-product facility strategy relies on a certain level of synergy between the manufacturing processes of each of the products for which the facility is intended to be used to produce. It would be impossible to develop a manufacturing facility capable of

housing and operating every possible purification unit operation in every conceivable configuration. As a result there must at least be some commonalities between the processes used to produce each product in order to make the use of a multi-product facility feasible. Fortunately, mAbs are ideally suited to being manufactured in such a facility as the platform approach, based on the use of Protein A chromatography, allows a process template to be applied to almost all mAb manufacturing processes. As has been previously described, this process template contains an upstream mammalian cell culture process, followed by primary clarification, product capture, intermediate purification, polishing and finally formulation. Primary clarification will usually be carried out using either centrifugation or dead-end filtration, possibly a combination of both. Meanwhile product capture, intermediate purification and polishing will usually be accomplished using some form of packed bed chromatography, and whilst the mode of chromatography used (e.g. Protein A, CEX, AEX HIC etc.) may vary from product to product, the equipment required to operate these steps will remain fairly consistent. As a result, whilst the use of the platform approach does not lock the process into any one particular configuration, it does provide a structure against which the multi-product facility may be developed, regardless of whether one is being built from scratch or if an existing facility is to be converted to produce multiple products. In the latter case, expansion of the facility will, in all probability, also be unnecessary since the amount of material which would need to be processed would be likely be less that 10kg per batch, a level of productivity which, as described, may be considered well within the productivity constraints of the majority of modern day commercial mAb manufacturing facilities.[9]

Based on this, it would seem that flexibility is a key consideration for multi-product facilities. Whilst the mAb purification platform does provide a basic structure for the manufacturing process, being biological molecules, there will always be subtle differences in the behaviour of different antibody products. Such differences may require adjustments to be made to the basic mAb manufacturing platform, such as the insertion or removal of process steps. They may also necessitate the re-ordering of steps within the purification train for example, or the changing of the modes of chromatog-

raphy used. The multi-product facility must not only be amenable to such changes, allowing equipment and plumbing within the facility to be altered to suit the product being manufactured, but must also allow these changes to be easily accommodated, without the need for excessive turnaround times between manufacturing campaigns.

Whilst the idea of multi-product facilities is based upon the need to produce smaller quantities of a particular mAb, it should be noted that the overall amount of product manufactured by the facility could still reach into the multi-ton range, depending upon the number of manufacturing campaigns being operated. As a result, the capacity limitations imposed by the use of packed bed chromatography processes, crucial in allowing the use of the platform approach to mAb processing, may still need to be considered. For example a single facility may be used to manufacture 5 different antibody products per year. If the annual product demand for each of these antibody drugs is 300kg, then the annual production volume required of the facility will actually be 1.5 tons. Assuming that the multi-product facility, and in particular the downstream process housed within, was originally designed to provide such a level of productivity, then this should not be an issue. However if the facility is to be tasked with manufacturing an additional sixth and seventh mAb product then, unless the plant was heavily under-utilised to begin, it might be necessary to build additional capacity into the facility. Such a scenario is reliant on the fact that a biopharmaceutical company actually has more than 5 different antibody products, with an annual demand of 300 kg. No drug company is currently in such a position. However given the vast amounts of resources currently being devoted to the investigation of new mAb drugs and the number of new antibody drugs in development pipelines as shown in Figure 2.2, then it could be argued that it is only a matter of time before a company finds itself in such a circumstance.

2.2.3 Industry Trends

Whether the major future challenge for Biomanufacturing lies in the development of facilities with multi-ton capacities, or in those capable of producing multiple products is up for debate. It is most likely that it will ultimately be a combination of both. It

has been predicted that in the future, successful antibody manufacturing companies will only have between five to seven products which require ton scale manufacturing, and that the majority of their portfolios will consist of products will much more modest production demands, ranging between 50 to 500kg.[25] These companies will therefore have to effectively manage their manufacturing facilities in order to provide both high throughput production of their blockbuster drugs as well as economical manufacture of their lower-scale products. In both cases, the challenge is to ensure that manufacturing factors such as costs and capacity are not limiting the level of production, but rather demand.

Addressing such a challenge is not straightforward since a prerequisite for being able to precisely scale production in such a manner, is that the demand for the antibody drugs to be manufactured is known at the point in the development timeline when the manufacturing facility is to be designed and built. This may not necessarily be the case, particularly when considering the success and consequently demand for new antibody drugs is far from predictable. Furthermore, drugs may be found to be effective against additional indications, beyond those which they were originally intended which will cause production demands to vary from initial projections adding a further variable to the equation.

This uncertainty in the potential demand for a drug will have a profound impact upon the design of the manufacturing facility used to produce it. The key aim for a biomanufacturer, seeking to construct a new mAb manufacturing facility, is to anticipate potential future demands, and ensure that the size of the facility does not cause it to become a bottleneck for productivity. This very situation has been faced by biomanufacturers during the late nineties, where the case of the biotherapeutic Enbrel^{TM} gave rise to the industry wide held opinion that an international shortfall of bio-manufacturing capacity was imminent as noted earlier. This shortfall was based upon there not being sufficient fermentation capacity available to generate the required amount of material. In response to this perceived limitation, there was a building boom in manufacturing facilities, with plants housing multiple 10,000 - 20,000 litre bioreactors being constructed. Since then, these large bioreactors, coupled with the

increases in cell culture titre achieved over the past half decade, have meant that upstream capacity is no longer an issue. The potential capacity shortfall has, for reasons previously discussed, now shifted downstream.[8] Utilisation of a packed bed chromatography based platform antibody purification process, unquestionably places a cap on the maximum productivity of the manufacturing facility housing the process. The manufacturer therefore has a number of options available. Firstly they may over build their facility, ensuring an excess of capacity which may result in unnecessary capital expenditure. Alternatively they may build a facility, and then add in additional capacity through plant expansion, as and when it is needed. Such a strategy has the drawback in that expansion would require manufacturing in that facility to be halted. A newly constructed manufacturing facility can take up to 5 years to come on line.[8] Even if it is assumed that expansion would only take a fraction of this time, the loss of revenue as a result of not meeting market demands during this period would be substantial. Of these two options the former is the more attractive, but even then, the manufacturer must make a decision as to exactly how much excess capacity to build into the plant. In this regard, the current paradigm for downstream processing of monoclonal antibodies, built around the use of the packed bed chromatography which scale with the amount of material to be processed, will always in a sense, be playing "catch-up" with the growth of the mAb market and the advances seen in upstream operations.

Whilst upstream operations have the potential ability to increase production to meet demand without the need for facility expansion, downstream processing, due to the high reliance on packed bed chromatography, cannot do the same without compromising process productivity. Thus whilst currently not an issue, it is this discrepancy between the prodution rate capabilities between upstream and downstream which ultimately fuels the concerns surrounding the potential capacity constraints imposed by packed bed chromatography.

2.3 Addressing The Incoming Challenges

In light of the challenges of production capacity and process costs faced by biomanufacturers, engineers have begun to investigate a number of strategies which may help to address these issues.

2.3.1 Improving Current Technologies

One way of addressing the concerns surrounding the use of packed bed chromatography, and it could be argued that the one which has the most manageable impact upon the use of a mAb platform purification approach, is to simply improve the technology used to make the chromatography processes more effective and efficient.

Dynamic Binding Capacity

As detailed previously, the concerns surrounding the high cost and potential capacity bottleneck presented by downstream purification, is based upon the reliance of current mAb purification platforms, upon packed bed chromatography processes which scale with the amount of IgG to be purified, rather than the process volume. Whilst such a scaling regime is not necessarily in and of itself an issue, the fact that resin capacities have not increased concomitantly with the cell culture titres seen upstream, which in some cases have increased by several orders of magnitude is a problem, leading to the potential cost and productivity issues detailed previously in Section 2.1.3. In light of this, an obvious way of alleviating these issues is to improve the capacity of the chromatography resins used. However such improvements are by no means trivial accomplishments.

The discrepancy between product titre and resin capacity is nowhere more apparent that with the case of modern Protein A chromatography media, where, whilst upstream product titres have increased up to 50 fold, achievable dynamic binding capacities have remained within the range of between 30 to 40g/L resin for the past decade.[12, 26]

A two-part study performed on a range of commercially available Protein A affinity

resins, revealed that the majority of Protein A resins have maximum dynamic binding capacities ranging between 20 and 30 g/L resin.[12] Some resins displayed higher dynamic binding capacities, with Mabselect SuRe attaining a maximum dynamic binding capacity of 40g/L at residence times of greater than 6 minutes.[26], after which point no further increases in capacity can be achieved through reductions in the mobile phase flow rate. The breakthrough experiments used to determine the dynamic binding capacities of these resins utilised feeds with a maximum IgG concentration of 1g/L, and whilst it might be expected that higher titre feeds would result in increases in dynamic binding capacities, such increases would likely only be incremental, and not the "quantum leaps" in capacity required to completely eliminate the cost and productivity concerns associated with Protein A packed bed chromatography.

The data would therefore suggest that increasing the dynamic binding capacity over and above the 30 to 40g/L resin range would require measures beyond simply changing operating parameters such as the mobile phase flow-rate and the associated residence times during loading, and would instead necessitate the need to alter the characteristics of the Protein A resin itself. The theoretical maximum DBC which can be achieved with an agarose based Protein A resin has been calculated to be approximately 70g/L resin[27]. This calculation however is based on several assumptions, such as uniform particle pore diameters, which are unlikely to be realised with current resins. However such a figure would suggest that it should be possible to increase the typical DBCs which may be achieved by improving the resins by, for example, synthesising resin particles with more uniform pore size distributions. Other ways of improving resin capacity which are currently being investigated include; optimising the resin particle size, increasing the ligand density as well as optimising ligand orientation to improve accessibility and therefore mass transfer within the packed bed.

Resin Compressibility

Aside from improving binding capacities, the structural stability of chromatography resins, is another area whereby improvements could help alleviate the associated productivity issues of packed bed chromatography. The majority of current Protein A

resins, including Mabselect SuRe which is arguably the first choice for Protein A chromatography, utilise a agarose based backbone. Agarose resins are compressible in nature, exhibiting non-linear pressure-flow relationships and are susceptible to wall support related effects upon scale-up, whereby the loss of frictional support forces providing by the walls of the column housing the packed bed at larger scales can lead to excessively high trans-column pressure drops, which can not only compromise the integrity of the chromatography column itself, but also the skid used to operate the process. Thus whilst it might be possible to operate a small scale column at a linear velocity of 500cm/h, it may not be feasible to use the same velocity at pilot and commercial manufacturing scales. The situation is exacerbated by the increases in product titre which ultimately lead to the use of larger columns which must be operated at ever decreasing mobile phase flow rates, thereby detrimentally affecting the productivity of the chromatography process.

Not all commercially available Protein A resins are agarose based. Prosep A (Millipore, MA) and Poros A (Applied Biosystems, CA) utilise a porous glass, and polystyrene divenylbenzene backbone respectively. These materials are more rigid than agarose and as a result these resins may be operated at higher mobile phase flow rates. Unfortunately the increased rigidity comes at the cost of decreased particle porosity, and as a result these resins generally exhibit lower dynamic binding capacities than their agarose based counterparts.[12, 26]. Thus whilst higher operating flow rates may help to increase process productivity, these benefits could be compromised by the potential need for more process cycles. Alternatively process costs may increase due to the need for larger column volumes. Regardless, the development of resins capable of operation at higher mobile phase flow rates is a key area, where improvements could potentially help reduce concerns regarding the productivity constraints imposed by packed bed chromatography.

Resin Cleaning, Regeneration and Re-use

A key factor in helping to alleviate the cost burden imposed by the use of protein A chromatography resin is the ability to re-use resins. Whilst the cost of Protein

A chromatography resins may be perceived as being high, in the case of commercial manufacturing, these costs may be distributed over the number cycles for which the resin may be re-used. Thus whilst it costs 10,000 $/ Litre for Mabselect SuRe, assuming a lifetime of 200 cycles, the cost per cycle is actually only 50 $/ Litre/ cycle.

The lifetime of Protein A resins is ultimately determined by the ability of the Protein A ligand to withstand alkaline conditions. Sodium hydroxide is typically employed as a Clean In Place (CIP) reagent as its a fairly simple and therefore cost effective solution. However its use for the cleaning of Protein A chromatography columns is constrained to a certain extent by the fact that Protein A ligands may be damaged by the alkaline conditions imposed by the use of NaOH. Using NaOH at relatively low concentrations can help to reduce the level of damage, but this can also reduce the effectiveness of the cleaning step. Thus whilst the stability of the Protein A ligands may not be impacted by repeated cleaning cycles, the build-up of non-specifically bound species to the packed bed and the potential for batch to batch carry-over of impurities reduces the effective resin lifetime. Mabselect SuRe was developed as a direct response to these issues. The ligand utilised on Mabselect SuRe is an alkali-stabilised Protein A derived molecule, allowing CIP to be performed with sodium hydroxide concentrations from 0.1M up to 0.5M. Mabselect SuRe has been shown to maintain dynamic binding capacities over 200 cycles using 0.1M NaOH as a CIP solution, whilst no carry over of non-specifically bound host cell proteins was detected over 100 cycles. This data is presented to serve as an example, and the actual resin lifetime will vary on a case to case basis, depending predominantly upon the characteristics of the feed to which the Protein A column is exposed, however it can be envisaged that further improvements along similar lines would help to improve the process economics associated with the use of Protein A chromatography and hence alleviate process cost concerns.

2.3.2 Disposable Technologies

Strategies for improving process productivity do not necessarily need to be constrained to increasing the productivity of a single process batch. This is particularly true of

multi-product facilities where the overall productivity of the facility across manufacturing batches and campaigns is equally important. Plant turnaround time therefore becomes an important factor.

The use of disposable technology allows the time required to switch a manufacturing facility over from one campaign to the next to be minimised, as it eliminates the need for equipment cleaning. Furthermore if there are differences in the processes used to manufacture the next product moving through the facility, due to the lack of hard-piping, the use of disposable technology enables more rapid plant reconfiguration. Indeed this type of plant flexibility decreases process development burden for cases in which rigid adherence to a particular process platform is unfeasible. The use of disposable technology also has the potential for presenting cost savings as it removes the need for the development and validation of equipment cleaning procedures.

The use of disposable technology does however have some drawbacks. Firstly the security of supply for certain disposable equipment such as bioreactors and buffer storage bags is currently fairly low.[25] The scale of such equipment is also relatively low and most likely only suitable for generating material for Phase I and II clinical trials. Operation at larger scales, such as for commercial manufacturing, not only becomes technically unfeasible but also the cost of replacing disposable equipment between batches makes traditional equipment re-use more economically viable. The use of disposable technology therefore currently seems most ideally suited to pilot facilities, used to manufacture material for early clinical trial purposes. Such facilities will most likely be used to produce a wide range of different products per year, thereby exploiting the flexibility advantages inferred through the utilisation of disposables.

2.3.3 Process Intensification and Continuous Processing

It could be argued that the cost and productivity constraints imposed by packed bed chromatography may be alleviated to a certain extent through smart engineering and operating the chromatography steps in a manner so as to maximise the efficiency of the process. Such an approach could involve intensifying the way in which the chromatography process is run so as to minimise the duration of certain operating

steps which do not add "value" to the product. The ultimate extension of this concept being a completely continuous downstream process.

Dual Flow-rate Feed Loading

In a typical packed bed chromatography process, the step with the single longest duration is the feed loading stage. During loading, even if the mechanical stability of the resin back bone means that bed compression is not an issue, the maximum feasible operating fluid flow rate is still constrained by the required residence time to achieve an acceptable dynamic binding capacity.

Optimisation of the loading flow rate will typically require a trade-off between the two metrics which determine the productivity of the chromatography process; namely the capacity (i.e. how much product is bound to the chromatography column) and the process time. Assuming a recovery of 100%, process productivity may be expressed as shown in Equation 2.2

$$Productivity = \frac{Capacity}{ProcessTime} \tag{2.2}$$

High mobile phase flow-rates will result in shorter process times which will increase the productivity of the process, but such improvements may be compromised by the resultant decrease in binding capacity. Similarly, reducing the mobile phase flow rate will increase the binding capacity of the column, but will come at the expense of longer cycle times.

A proposed strategy for mitigating the magnitude of the trade-off between capacity and process time, and therefore increasing productivity, is to utilise two different flow rates during loading.[28] The strategy essentially involves operating the first stage of the loading process at a high flow rate, and then switching to a slower flow rate midway through the step. The rationale for this dual flow rate loading approach, is that at the beginning of the load step, the column is completely unsaturated and as a result there are plenty of ligand binding sites available to the product molecule. As a result residence time is not a hugely important factor during this early stage of

the loading step and the column can be operated at high mobile phase velocities. As the loading stage progresses, the column becomes more saturated with product, and ligand binding sites become less readily available. At this point the mobile phase flow rate is reduced, allowing product molecules time to penetrate deeper into the pores of the resin and find the remaining ligand binding sites, thereby maximising column capacity utilisation. Operating the initial part of the loading step at a high mobile phase flow rate reduces the total duration of the loading step, thereby reducing cycle times, whilst lowering the flow rate during the second half of the loading step helps to maintain binding capacities at an acceptable level. Such an approach requires optimisation of the two flow rates used and also the point during the loading step at which the switch in the flow rate is made.

Simulated Moving Bed Chromatography

Process intensification as a means for improving efficiency and productivity is based upon the concept of minimising the duration of process steps which do not in and of themselves, add "value" to the product. An example of this would be the cleaning and regeneration steps performed at the end each chromatography cycle. Hold times between process steps may also be considered to be in this category and as a result the ultimate extension of this concept would be a completely continuous downstream process, in which equipment utilisation and productivity are both maximised.

Unfortunately, the manner in which conventional packed bed chromatography processes run in bind and elute mode, are operated, makes implementing a truly continuous processing strategy difficult. Columns must be loaded, washed and then eluted in a stepwise manner. However work has been performed towards developing chromatography processes which may be more amenable to continuous processing.

In a simulated moving bed chromatography system, multiple chromatography columns operated in bind and elute mode are utilised, such that the individual steps making up a complete chromatography cycle (e.g. equilibration, load, wash, elution and strip) on each column are staggered. Doing so allows the chromatography system to be operated in a continuous manner such that whilst one column is being eluted,

the next may be equilibrated and loaded with feed material.

The simplest form of simulated moving bed, is a three column periodic counter current chromatography system. As the name suggests, the system utilises three chromatography columns operated essentially in tandem as shown in Figure2.11.

The system shown in Figure2.11 is comprised of three identical chromatography columns denoted A,B and C. These column take up positions labelled 1, 2 and 3 respectively. Position 1 may be thought of as the load position. The column in position 1 will be directly loaded with the feed material for the counter current chromatography step. Position 2 meanwhile may be thought of as the secondary column. The column in this position will be used to capture the product in the flowthrough from the column in position 1. Position 3 is the recovery position. The column in this position will undergo wash, elution and regeneration steps in order to recover bound product and also return the column to a state ready for feed loading.

The operation starts with the equilibration and loading of Column A at position 1. The flowthrough from Column A is then loaded directly onto Column B at position 2. The "catching" of the flowthrough from Column A on Column B allows the first column to be loaded to a breakthrough level well beyond that which might typically be used for a chromatography process, since any product not bound on Column A will be captured on Column B. Increasing the breakthrough percentage allows the resin utilisation to be maximised which helps to improve the economic efficiency of the process. Once Column A is sufficiently saturated with product, it is moved from position 1 to position 3, whilst Column B and Column C move to positions 1 and 2 respectively. The load is then switched to Column B with the flowthrough loaded directly onto Column C. Meanwhile Column A undergoes wash, elution, strip and regeneration steps, preparing it for re-use. Once Column B is saturated it is switched to position 3, whilst Column C moves to position 1 and Column A moves to position 2. Loading is then continued on Column C with the flowthrough collected on Column A. Once column C is saturated it is moved to position 3, Column A moves to position 1 and Column B moves to position 2. At this point the three column have been returned to their initial positions and the cycling of columns may be continued until

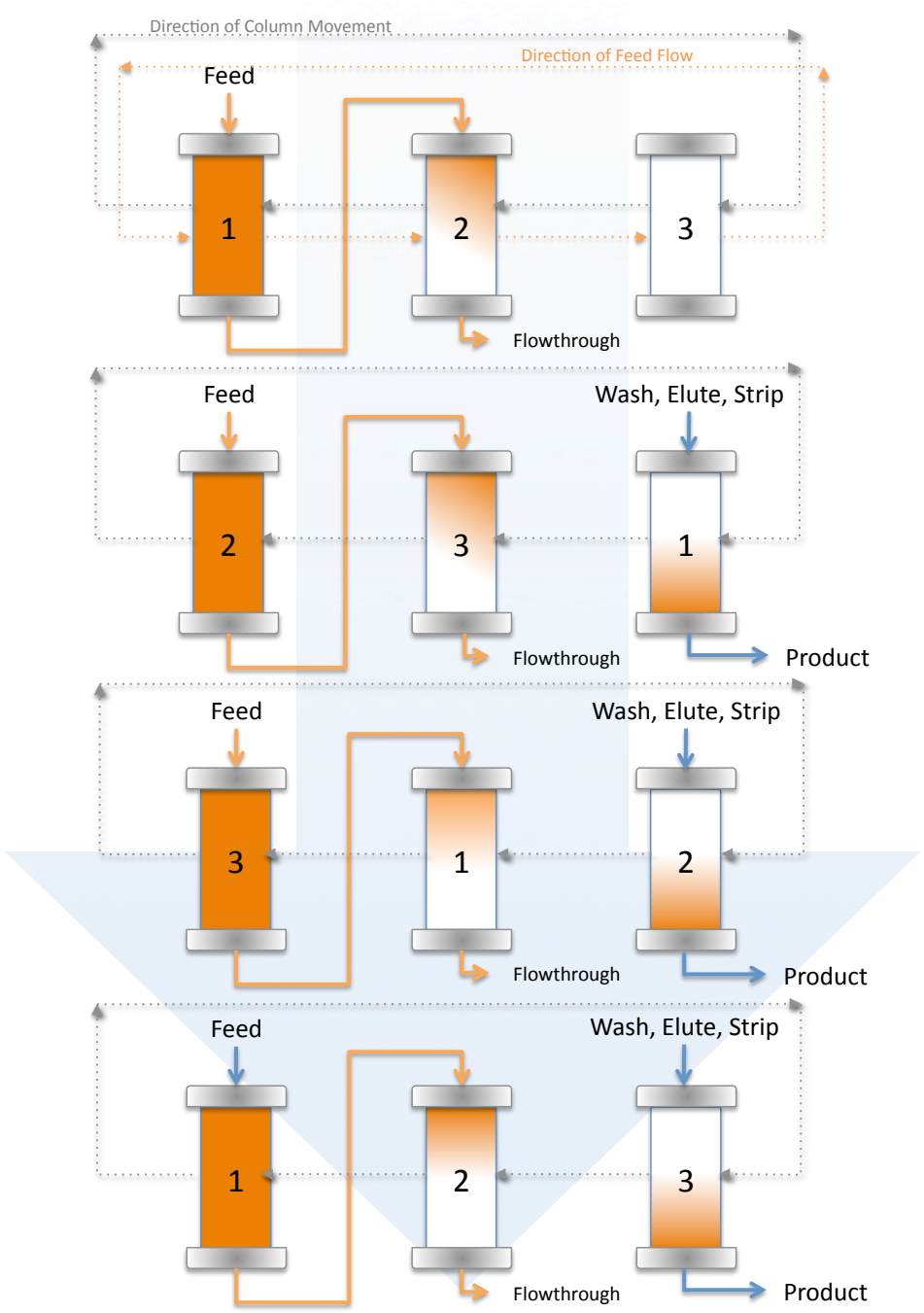


Figure 2.11: Schematic showing operation of a 3 column periodic counter current chromatography system

all the feed has been loaded. Whilst such a system is clearly amenable to continuous processing, it also present benefits if operated in a batchwise manner. As previously alluded to, the loading of each of the columns to high breakthrough percentages, maximises the resin utilisation which can reduce the cost of good for the process. The staggering of the chromatography cycles also helps to reduce process times which along with the high resin utilisation can also help to increase the overall productivity of the chromatography process.

The one major drawback of adopting such a system, is the increase in operational complexity. Effective operation of the counter current system requires accurate control of valve switching so that columns can shift positions at the required points during the process. This in turn requires simultaneous monitoring of three separate interacting chromatography columns which are essentially being operated in parallel. Process deviations would be difficult to handle in such a situation, as if an unexpected event were to occur on one column, it would have implications as to the operation of the other columns in the system.

2.3.4 Alternative Bioseparation Technologies

Whilst the previously outlined strategies for improving the productivity and economics of MAb manufacturing are all centred around optimising and augmenting the currently accepted Protein A based process platform, another proposed strategy involves a slightly more radical approach, in which unit operations within the platform are replaced with so called "alternative" bioseparation techniques. This strategy is based upon the concept that the cost and productivity constraints of current MAb purification platforms are inherent due to the high dependence upon packed bed chromatography operations which scale with the mass of product to be purified rather than the process volume. Thus whilst these constraints may be relaxed through the previously outlined approaches, they may only be truly overcome if the dependence upon packed bed chromatography can be reduced or even eliminated altogether. It has therefore been proposed that by replacing certain packed bed chromatography steps with these alternative bioseparation technique, which have the potential to pro-

vide higher productivities at reduced costs, it may be possible to mitigate the risk of downstream processing to become a capacity bottleneck as a result of the increases in upstream titre.

Whilst these techniques may indeed offer benefits either in terms of process productivity or the cost of goods, their adoption also has the potential to disrupt current MAb purification platforms which are built upon the utilisation of packed bed chromatography processes. For example,whilst many of the alternative bioseparation techniques which have so far been proposed are less expensive to operate and/or offer higher productivities, they are unable to match the level of robust purification afforded by Protein A chromatography. As previously detailed, it is indeed this level of robustness which actually allows the adoption of a platform approach to the purification of MAbs in the first place. It would therefore seem that the replacement of Protein A chromatography with an alternative bioseparation technique would potentially require a dramatic shift in the current MAb purification paradigm and movement away from this templated approach to the purification of antibody products. The consequence of such a shift would be an increased burden on process development. It could be argued that this is the price which must be paid for the increased productivity or reduction in operating costs afforded by the use of alternative bioseparation techniques. Considering the often stringent time and resource constraints imposed upon process development, it is not surprising that it is a price that many perceive to be too much to pay. However such perceptions are based on short term considerations and may not necessarily reflect the long term benefits which may be received from the utilisation of these alternative techniques. Whilst it is true that none of these alternatives are currently capable of matching the purification performance and robustness of certain packed bed chromatography processes, this does not necessarily mean that this will always be the case. Technological developments are ongoing with many of these emergent techniques. And even if the use of alternative techniques results in increase development costs, these costs may be potentially justified in the long term by the costs saved by de-coupling the facility size from the production capacity during commercial manufacturing. It is indeed this potential for moving away from

the current purification paradigms, so constrained by the high reliance on packed bed chromatography, which present such a compelling case for at least the investigation if not the adoption of these alternative bioseparation techniques.

The remainder of this thesis will be focused on the evaluation of these alternative techniques, with the aim being to determine quantitatively the relative advantages these techniques present over conventional packed bed chromatography. Efforts will also be made to discern the major drawbacks and limitations associated with each of these techniques. Such investigations should provide insights as to how these alternative bioseparation techniques may be incorporated into current biopharmaceutical manufacturing processes. To begin, the following chapter outlines some of the alternative bioseparation techniques which have been proposed, with details provided regarding the mechanisms of separation utilised (e.g. size, charge, hydrophobicity etc.), the potential benefits provided as well as major drawbacks of each technique.

Chapter 3

Alternative Bioseparation Techniques

3.1 Introduction

The term "alternative bioseparation technique" is fairly ambiguous and depending upon the context it which it is used, may be interpreted to include any number of bioseparation processes. In terms of this body of work, the term refers first and foremost to non-packed bed chromatographic techniques which are able to provide some degree of selective purification of biomolecules. The term does not include any techniques which may be regarded as traditional or conventional unit operations such as ultrafiltration and centrifugation. Under this definition a number of alternative bioseparation techniques have recently been proposed, each utilising different mechanisms of separation and offering different levels of purification. What these techniques all have in common however is the potential for high process productivity or capacity at a possibly reduced cost compared to conventional packed bed chromatography.

Recent reviews have briefly outlined the concepts behind some of these alternative techniques, and the potential benefits which they may provide.[29, 11, 27] In this chapter, details are provided on a selection of these techniques. The list of alternatives which are described is not intended to be completely comprehensive. Given the recent level of interest in this area of biomanufacturing, it is likely that new separation technologies

are being proposed on a daily basis and as a result compiling an exhaustive list of all current alternative techniques would be difficult. Instead the aim is to provide a degree of insight into the techniques which have garnered the most interest or show the most potential for adoption into biomanufacturing processes. Details are provided on the mechanisms of separation being utilised by some of these techniques, the potential technical and economical advantages which they may provide and also the possible drawbacks associated with their adoption. The purpose of this is to develop a greater knowledge and understanding of some of these techniques, which may then be used as a foundation upon which to perform quantitative evaluations in order to determine their potential for using in biomanufacturing.

A few of the separation technologies which have been outlined are not necessarily novel or emergent. Indeed, techniques such as crystallisation and aqueous two phase extraction are relatively mature techniques. However it is as a result of recent advances which have lead to renewed levels of interest in these alternatives. In the case of aqueous two phase extraction, it is the development of cheaper polymer systems with the potential for polymer recycling which have made the use of this technique at large scale slightly more feasible. With crystallisation, the increased titres produced upstream have made bulk protein crystallisation as a purification technique a possibility.

The techniques described may be broadly categorised into three main groups. Bulk separation techniques involve addition of components to the product pool, causing some form of phase change. For example the product may move from being in solution to forming a solid phase, which is the case in precipitation and crystallisation processes, both of which are therefore included in this group. Other techniques include Aqueous Two Phase Extraction (ATPE) and Three Phase Partitioning (TPP).

Field-based separation techniques is another group of alternatives. These techniques utilise imposed force fields to affect bioseparation. These fields could be flow, magnetic or electric. Techniques included in this group are High Performance Tangential Flow Filtration (HPTFF) which can use charged membranes to enhance TFF performance as well as Controlled Shear Affinity Filtration (CSAF)

The final group of alternatives are adsorptive separation techniques which rely on sorbent-sorbate interactions. These techniques achieve purification through binding of feed components, be it either product or impurities, using ligands immobilised to a suitable stationary phase. This is essentially the concept behind conventional packed bed chromatography and as a result, techniques included in this list are essentially chromatographic operations utilising alternative forms of the stationary phase, such as membranes and monoliths, which allow for higher throughputs.

The following is a brief description of each of these groups and some of the alternative techniques of which they are comprised.

3.2 Bulk Separation Techniques

Bulk separation techniques involve the movement of process stream components from one phase into another. Phase separation techniques are then used into order to affect bioseparation. All of these techniques involve the addition of materials to the bulk processing fluid, causing either a change in the physicochemical environment and/or the activation of affinity based interactions. This then leads to the aforementioned component phase change. The techniques reviewed in this section include:

- Aqueous Two Phase Extraction (ATPE)
- Primary and Secondary Effect Affinity Precipitation (PEAP & SEAP)
- Three Phase Partitioning (TPP)
- Macroligand Facilitated Three Phase Partitioning (MLFTPP)
- Crystallisation (Cryst)

All bulk separation techniques share the potential advantage of scaling with the process volume rather than the mass of product to be purified. This mitigates the risk of such techniques straining plant space constraints, in the manner that packed bed operations would, as a result of increasing upstream titres. For example if a 1,000L fermenter were to express product at a titre of 1g/L, the required precipitation tank

could still be used to process the material from the same fermenter even if the titre were to increase to 10g/L, since the process volume remains constant even if the product mass increases. It is for this reason that some biochemical engineers see the use of such bulk separation techniques, particularly as early capture steps, as a way of reducing the potential capacity constraints imposed by downstream processing.

3.2.1 Aqueous Two Phase Extraction

Introduction

An aqueous two phase extraction (ATPE) system is made up of two discreet aqueous solutions of either a polymer and a lytropic salt or two different polymers. When solutions of the polymer and salt or of the two different polymers are mixed, two discreet phases will form. Each phase will contain a different %w/w of each polymer (or polymer and salt, if a polymer/salt system is used), and as a result each phase will have a different density. Left to settle under gravity, the two will separate into a top and bottom phase. ATPE works on the principle that a target species, when introduced to such a system, will partition at different concentrations into the two different phases. The use of an aqueous environment differentiates this technique from organic solvent extraction, since it presents the target molecule with an environment more suitable for biologically active substances. This makes ATPE particularly attractive when the target species is a protein which may become denatured when using any type of solvent extraction.

Conventionally PEG/dextran or PEG/potassium phosphate systems have been used, however whilst yielding good separation results, both systems have either been too expensive to use in an industrial/commercial context (in the case of PEG/dextran systems, due to the high cost of dextran), or have presented processing problems (high salt concentrations used in PEG/potassium phosphate systems can lead to protein denaturation). Recent advances however have led to the development of less expensive ATPE systems, which are easier to process. For example, the use of thermoseparating polymers such as Ethylene oxide-propylene oxide (EOPO), or pH sensitive polymers

such as polydiallyaminoethanoate-dimethyl sulfoxide, which offer the advantage of easy back extraction of the target species by either raising the temperature or changing the pH to cause polymer precipitation.

Operation of an ATPE Process

ATPE is usually performed on a bulk fluid. The feed is held in a suitable container and the components of the two phase system are then added. The feed and the components of the two phase system are then mixed in order to aid mass transport, and to allow the components of the feed to partition into the appropriate phases. The two phases of an ATPE system are commonly referred to as the top and bottom phases. In a polymer/ salt system, the top phase will normally be the aqueous polymer rich phase, whilst the bottom phase will be the salt phase. In a two polymer system, such as a PEG/Dextran extraction, the aqueous PEG will form the top phase, whilst the Dextran rich solution will form the bottom phase. ATPE systems will typically be developed such that, once the process feed is introduced, the product will preferentially partition to the top phase whilst impurities (e.g. DNA, HCP and cell debris) will partition to the bottom phase. However the exact partitioning behaviour of the product will depend upon a number of factors including the physicochemical characteristics of the product and impurities. As a result the degree of control over which phase the product moves to can often be limited and in many cases the product could partition to the bottom phase with impurities moving to the top phase. Once adequately mixed and following a suitable incubation period to allow for mass transfer between the two phases, the two phases must be separated (for example using centrifugation). Once the product enriched phase has been recovered, the next step is to back extract the target biomolecule. The method of back extraction will to a certain degree depend upon the phase into which the majority of the product has partitioned. For example if a polymer/ salt system were used and the product partitioned into the bottom salt phase, back extraction of the product would be straightforward. The bottom salt phase could simply be loaded onto a chromatography column and the product could then be eluted (assuming that the conditions of the bottom phase are compatible with the mode of chromatography

being used). However if the product had partitioned into the top polymer phase then extraction is complicated somewhat. In some processes it may still be possible to directly load the top phase onto a chromatography column and elute the product off.[30] However in the majority of cases, back extraction has been traditionally done by the addition of a salt to the polymer solution to form a new aqueous two phase system. By altering the physiochemical environment it may then be possible to cause the target biomolecule to concentrate into the bottom salt phase. Back extraction of the product from an aqueous polymer rich phase, is one of the major bottlenecks of any ATPE process, and a large amount of research has gone into finding ways of making it easier to recover the target biomolecule from an aqueous polymer phase. One example is the development of stimuli responsive polymers which react to changes in the physicochemical environment. Such polymers will precipitate out of solution upon receiving particular stimuli, which may be for example an increase in temperature (Thermoseparating Polymers) or a change in the pH (pH responsive polymers). In terms of operating costs, a major cost factor will be the polymers used in an ATPE system. Ideally, once the target biomolecule has been back extracted from the aqueous polymer, it should be recycled in order to reduce costs. Studies have shown that it may be possible to recycle the same aqueous polymer over up to four cycles (three reuses) without affecting the performance of the ATPE step.[31] Figure 3.1 summarises the typical stages present in an ATPE process being used to capture and concentrate a product biomolecule from crude fermentation broth.

Key Parameters for Process Development

The Binodial Curve - ATPE systems are formed by mixing aqueous dispersions of two incompatible polymers or a polymer salt interaction. Such systems can be represented by a binodial curve phase diagram, a schematic of which is shown in Figure 3.2.

The binodial curve is the border between the one phase and two-phase regions. Mixtures formed at polymer compositions above the line will form the two phase systems required for ATPE. Polymer mixtures at compositions below the line will form monophasic systems. The dotted tie lines shown in Figure 3.2 represent the

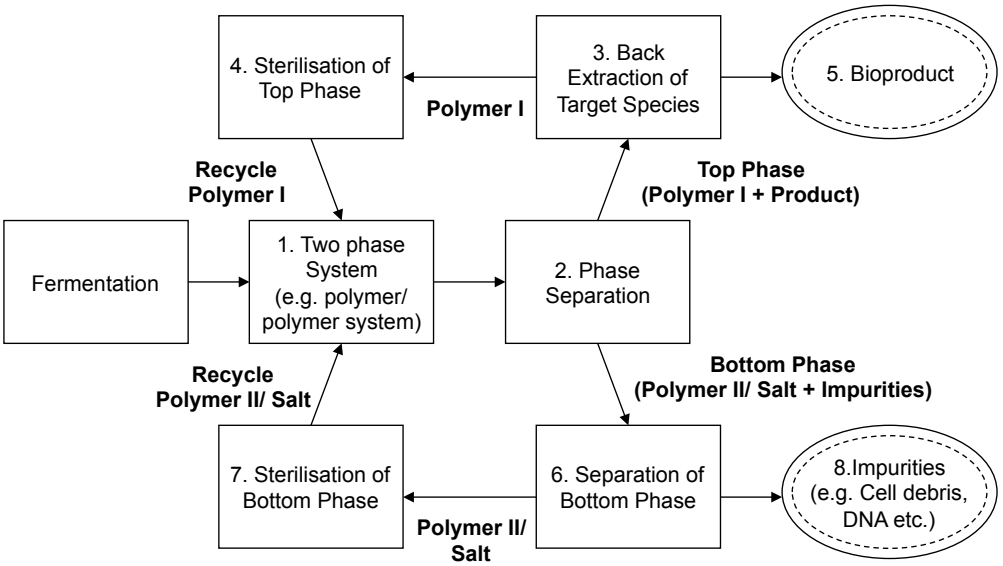


Figure 3.1: Schematic showing the typical steps involved in an aqueous two phase extraction process.

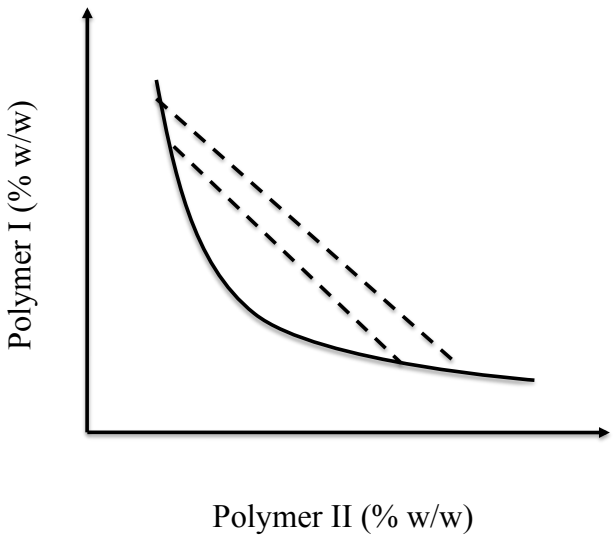


Figure 3.2: Schematic of a typical binodial curve phase diagram

conditions in the top and bottom phases of the two phase system at equilibrium. Any system prepared along the same tie line will result in the same top and bottom phase conditions; however the volume ratios of the two phases will change. The binodial curve essentially places a limit on the lowest concentrations of polymer required in order to generate a two phase system. At large scale, the lower the amount of polymer required in order to generate a two phase system, the easier the system becomes to operate and also the more cost efficient the process becomes. Studies have shown that the binodial curve can be shifted towards lower polymer concentrations through increasing the molecular weight of the polymers and also through the addition of salts.[32] The addition of biomass, such as when introducing a process feed to the two phase system, will also result in a shift of the binodial curve towards lower polymer concentrations.

Partition Coefficient - A key parameter when dealing with ATPE systems is the partition coefficient (K), which is essentially a measure of the concentration of a particular molecular species in the top phase expressed as a fraction of that present in the bottom phase, as shown in Equation 3.1

$$K_A = \frac{\text{Concentration of A in Top Phase}}{\text{Concentration of A in Bottom Phase}} \tag{3.1}$$

Based on this, the aim would be to develop the ATPE system such that the product has a high partition coefficient, whilst impurities have a very low partition coefficient (or vice versa). Recovery of the product enriched phase would then allow purification.

Phase Ratio - Another important parameter when dealing with ATPE systems is the phase ratio. This will directly impact the product recovery achievable with an ATPE system. The phase ratio is usually defined as the ratio between the volume of the top phase (V_t) to that of the bottom (V_b).

$$\text{Phase Ratio} = \frac{V_t}{V_b} \tag{3.2}$$

If the product is being recovered in the top phase then a high Phase ratio would be desirable, whilst if the product is to be recovered in the bottom phase, then a low

phase ratio would be required in order to maximise the yield achievable using the ATPE process. As previously alluded to, the phase ratio can be influenced by careful selection of the operating point on the binodial phase diagram.

A number of factors will affect the partitioning behaviour of a particular biomolecular species and hence the performance, in terms of yield and recovery, of an ATPE process. These factors include the properties of the target molecule (net charge, hydrophobicity/hydrophillicity), the properties of the polymer (molecular weight, hydrophobicity, charge) and the physicochemical conditions of the system (pH, ionic strength and temperature).[33] These factors will combine to influence the partitioning behaviour of the product molecule and as a result can be taken advantage of in order to optimise the performance of any ATPE operation. Given that the performance of the ATPE process is dependent on such a wide range of interacting parameters, development and optimisation of ATPE processes would most likely require the use of some form of high thoughput screening methodology.

Technological Developments

As stated previously, back extraction of the target biomolecule from an aqueous polymer phase is one of the major bottlenecks of any ATPE process, in which the product partitions into a Polymer phase. Recent advances have however meant that the process of back extraction has been simplified somewhat through the use of specially designed/ selected polymers. Thermoseparating polymers (such as the random copolymer of 50% (w/w) ethylene oxide and 50% (w/w) propylene oxide $EO_{50}PO_{50}$) react to changes in temperature.[31] When such polymers, in solution, experience an increase in temperature a new phase system forms. One phase, usually the bottom phase will be enriched with the Thermoseparating polymer, whilst the other phase will be depleted, the principle being that if the original aqueous polymer solution contained the product biomolecule, this would partition into the polymer depleted top phase. Separation of the product from the polymer can then be achieved through a centrifugation process. Similar in principle to Thermoseparating polymers, pH responsive polymers will also form two phases, upon changes in pH. One drawback of

ATPE is its level of selectivity. Whilst it can affect some resolution between different types of molecules, for example separation of proteins from cell debris and nucleic acids, ATPE systems have a greater level of difficulty resolving different molecules of the same type. Affinity partitioning has been envisioned as a way of improving the selectivity of an ATPE process, through the utilisation of an affinity ligand, which has specific interaction with the target molecule of interest. By coupling this affinity ligand to one of the phase forming polymers it would, in principle, be possible to influence the partitioning behaviour of the feed components. There have been examples in which ATPE systems have been used with hydrophobic affinity ligands coupled to one of the phase forming polymers. These ligands interact with hydrophobic sites which exist on the surface of the biomolecules present in the feed, providing separation selectivity for molecules with hydrophobic surface residues.[34] There have also been examples of ATPE systems which utilised biospecific affinity ligands.[35] The coupling of such ligands to a phase forming polymer allows a very high level of selective separation to be achieved. Such a technique would be very useful if poor partitioning of all feed components towards a particular phase is seen, since attachment of a biospecific ligand with and affinity for the target biomolecule of interest, to such a phase would mean that a high product resolution could be achieved.

Potential Applications

Aqueous two phase extraction has been used as a preparative and analytical tool (e.g. to determine protein structure since partitioning is dependent on protein surface hydrophobicity). However the ease at which processes can be scaled, means that it may be effectively used as an early selective separation step, where the volumes of material which need to be handled is large. ATPE can also handle the processing of crude feedstocks. For example there have been several cases in which ATPE has been used to capture and concentrate a product rProtein from crude fermentation broth containing whole cells, cell debris and nucleic acids.[36, 37, 30, 38, 32] When a crude feed stock is introduced to an ATPE system, the rProtein partitions into the top phase whilst cells, cell debris and nucleic acids move into the bottom phase. The ability of

ATPE to be used to process large volumes of crude material whilst affecting a certain degree of separation selectivity is its major advantage, making ATPE an ideal first step in a downstream processing train. ATPE has been, in numerous cases, shown to work as a primary recovery process and has also been effectively integrated into fermentation processes, allowing for *in-situ* recovery of a target protein.[33] Studies have also been conducted on the use of ATPE to capture and purify nucleic acids. More specifically, ATPE has been used to purify recombinant DNA plasmids from cell lysates, obtained from preceding alkaline lysis and centrifugation steps.[32] In this study, ATPE was used as a intermediate purification step, used to remove a significant amount of contaminating host cell proteins and RNA, reducing the purification burden placed on subsequent downstream processing steps.

Summary

ATPE is not a total solution to protein purification since it is not sufficiently selective.[39] Advancements in polymer technology may result in an improvement in the partitioning of product biomolecules and hence the resolution achievable using ATPE. However is likely that high resolution separation processes such as chromatography will be required in order to provide the level of purity which will be needed for biopharmaceutical products. However due to the ease of scalability and the degree of selectivity which is offered, coupled with its ability to handle bulk crude feed stocks, ATPE can be used effectively to combine a number of initial downstream purification steps including clarification, product capture, concentration and primary purification, thereby reducing the number of downstream processing operations and also the purification burden placed on subsequent steps. Typical protein purification factors achievable using ATPE range between 1 and 9 fold, with an average of approximately 3-fold. ATPE also has a relatively high yield with an average of approximately 85% across all of the literature reviewed. In terms of cost, ATPE can require a relatively high capital expenditure due to the fact that several unit operations are required in order to run an entire ATPE process. Additional capital expenditure will be needed if the phase forming polymers used are to be recycled. In terms of operating costs, the

actual cost of goods may be relatively low, with the main cost driver being the phase forming polymer(s) required for the ATPE system. These costs may be increased if biospecific affinity ligands are utilised to improve the selectivity of the process. These characteristic all combine to suggest that ATPE would be ideally suited for use as the primary downstream step in a protein purification process.

3.2.2 Affinity Precipitation

Introduction

The basic principle behind bioseparations through precipitation is that through a change in a crucial physicochemical parameter, the solubility of either the target molecule for purification or impurities can be sufficiently reduced that the component comes out of solution forming an insoluble precipitate. Recovery or removal of this precipitate may then enable bioseparation. Protein precipitation has been used in industry as an early product capture step, however its lack of specificity has limited its use somewhat. As a result efforts have been made towards increasing the selectivity of precipitation operations, through the incorporation of affinity technology, which is so effectively utilised in affinity chromatography. The affinity ligand can be a biological ligand (effectively utilising enzyme-substrate interactions) or they can be other types of affinity ligand (e.g. metal ions). This has led to the development of two types of affinity precipitation processes, namely primary effect affinity precipitation (PEAP) and secondary effect affinity precipitation (SEAP).[40] These two techniques differ in the mechanisms in which precipitation of the product biomolecule is brought about.

Primary effect affinity precipitation - In primary effect affinity precipitation, the interactions between the target molecule and its affinity ligand cause the formation of the precipitate. An example of this is the reaction which occurs between the enzyme, lactate dehydrogenase (LDH) and bifunctional N_2,N"$_2$-adipodihydrazido-bis-(N_6-carbonyl-methyl-NAD) (Bis-NAD) to which it has an affinity.[40] When LDH and Bis-NAD are mixed together at a certain concentration ratio, they form a crosslinked, macromolecular network which eventually becomes insoluble and precipitates out of

solution when the aggregates become sufficiently large. Thus the affinity interaction between the protein target and the affinity ligand and precipitation of the formed ligand-protein complex is linked. The efficiency of this type of affinity precipitation is highly dependent upon the ratio of concentrations of the affinity and target molecules. Small deviations from the optimal concentration ratio can lead to drastic reductions in the level of precipitation observed.[40] As a result, primary effect affinity precipitation processes have tended lack robustness, especially at process scale where fairly elaborate pilot scale tests are required in order to determine the optimal conditions for precipitation.

Secondary effect affinity precipitation - In secondary effect affinity precipitation, protein-ligand interaction and precipitation are no longer linked and as a result can be controlled independently. By uncoupling the two mechanisms, secondary effect affinity precipitation becomes more flexible and robust. For example secondary effect affinity precipitation processes have shown a much greater level of tolerance to variations in process parameters, without a change in process performance.[40] As with primary effect precipitation, secondary effect precipitation also exploits the biospecific interactions between a protein and a ligand to which it has an affinity. However with secondary effect affinity precipitation the ligand is found normally conjugated to a polymeric substance. A number of poly-N-akylacrylamides have successfully been employed for this purpose, and represent a typical polymer which is used. The ligand-polymer agent is called an Affinity Macro-Ligand (AML) and will have the characteristic that it can be made to reversibly precipitate out of an aqueous solution upon receiving an external stimulus (such as a change in temperature or pH). Thus in a secondary effect affinity precipitation process, the first step will involve getting the AML to interact with the target protein, forming an AML-protein complex. Once this reaction is complete, the required stimulus to cause the AML to precipitate can be introduced, causing the AML to effectively "pull" the target protein out of solution. Secondary effect affinity precipitation presents an attractive downstream processing option since it combines the well known process of precipitation with the specificity of an affinity approach. It has the advantage over primary effect affinity precipitation in that it is

much more robust and because the affinity reaction and the precipitation reaction are uncoupled, is much more flexible. Indeed, secondary effect affinity precipitation has been ranked among the highest of affinity based separation techniques, in terms of its resolving power and also its large scale potential.[41]

Of the two forms of affinity precipitation, secondary effect affinity precipitation shows greater potential for adoption at large scale. As a result the remainder of this section will only detail secondary effect affinity precipitation.

Operation of an Affinity Precipitation Process

A major factor affecting the effectiveness of a secondary affinity precipitation process, is the choice of AML which is used. Once the polymer has been chosen, the ligand, for which the product protein has an affinity for must be coupled to the polymer in order to form the AML. When synthesising the AML, due to the fact that the affinity ligands are integrated statistically all over the polymer backbone, a certain degree of heterogeneity may be observed with regards to the characteristics of the individual AML molecules. Also the affinity of the ligand is generally reduced upon coupling to a polymer, by up to a factor of 1000.[40]

Affinity precipitation is a bulk separation technique, meaning that the AML, in solution is simply introduced to the process feed in a container (for example a tank) and left to incubate whilst being agitated. Once the affinity interaction between the product and the AML is complete, the next step will be to introduce the external stimulus which will cause the AML to precipitate out of solution, drawing the product along with it. Once the AML precipitates, it can be recovered using some form of solid-liquid separation process (e.g. centrifugation). The supernatant from such a process can be disposed of, whilst the precipitate must be resolubilised and washed. The method of precipitate resolubilisation will depend to a certain extent on the stimuli to which the AML responds. For example if it is a thermoresponsive AML, the precipitate may be resolubilised by lowering the temperature.

Back extraction or elution of the product from the AML-product complex can be achieved in one of two ways. The most straight forward way is to immerse the

precipitate in a buffer solution and provide a physicochemical environment which maintains the AML as a precipitate, but also disrupts the affinity interaction between the affinity ligand and the product. Such interactions usually require a change in the environmental conditions (e.g. ionic strength, pH, temperature etc.). The product is essentially eluted from the precipitate into the extraction buffer. The conditions used for product elution will depend upon the AML which is used and the external stimuli required in order to cause its precipitation. An alternative way of eluting the product is to initially, completely resolubilise the precipitate (including the AML) by removing the external stimuli causing precipitation. Once this has been done, the physicochemical environment can be manipulated in order to disrupt the interaction between the affinity ligand and the product. Whilst maintaining this "disruptive" environment, the external stimulus which causes precipitation of the AML can then be reintroduced. This will in turn cause the AML to come out of solution, whilst the product remains in solution.

Once the product has been eluted from the AML, and the AML has been recovered, the next step will be to recycle the AML leaving it ready for another round of affinity precipitation. Given the fact that the AML contains an affinity ligand and also that quite elaborate chemical reactions may have been required in order to synthesise the AML, recycling may provide a substantial cost benefit. It has been shown that AMLs may be reused up to four times without a drop in the purification performance.[42] The schematic diagram shown in Figure 3.3 summarises the steps which are typically required to run a complete secondary effect affinity precipitation operation (as indicated by the steps enclosed within the dashed lines).

Key Parameters for Process Development

Process development of a secondary effect affinity precipitation process can be thought of in two parts. Firstly there is the development and optimisation of the affinity interaction process. Conditions must be chosen in order to maximise the strength of the interactions between the AML and the ligand. These interactions will determine the amount of product which can be captured by an affinity precipitation step, and

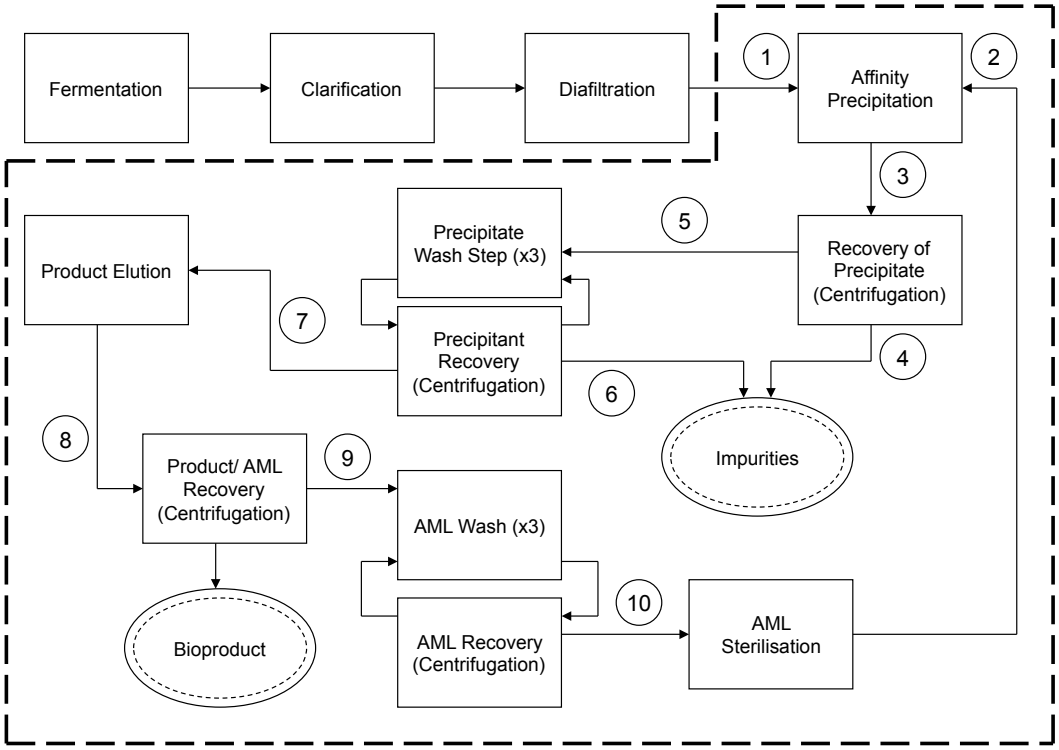


Figure 3.3: Schematic diagram showing the typical steps involved in a secondary effect affinity precipitation process (The steps which actually constitute the affinity precipitation process is enclosed within the dashed lines)
1. Feed - Following fermentation a clarification step will be required to remove whole cells and cell debris. *2. Affinity Macro-Ligand (AML)* - The AML is introduced to the feed. The external stimulus for precipitation can then be introduced. *3. AML-Product Precipitate* - The process fluid moves onto a solid-liquid separation step, in order to recover the precipitate product. *4. Impurities* - Impurities which are not bound to the AML or entrapped within the precipitate are removed from the process stream. *5.Precipitate and Entrapped Impurities* - Washing will essentially involve resolubilising and then reforming the precipitate. *6. Impurities* - Impurities initially entrapped in the AML-Product precipitate are washed out and removed from the process stream. *7. AML-Product Precipitate (Washed)* - The precipitate, washed of impurities moves onto the next step for elution of the product. *8. AML (Precipitate) & Product (Solution)* - Following elution the AML and product moves on to be separated via centrifugation. *9. AML (Solution)* - After being separated from the product, the AML may be washed and resolubilised in a suitable buffer. *10. AML (Binding Buffer)* - The AML is sterilised ready for reuse in the next precipitation cycle.

hence the process yields which are achievable. As a result high affinity constants from the AMLs are required ($K_D < 10^{-10}$). The affinity of the product to the AML will to a certain extent depend upon the affinity ligand which is used, however the choice of polymer to which this ligand is attached may also have an effect. Furthermore, manipulation of the physicochemical environment of the affinity precipitation system (pH, ionic strength, temperature) can help to optimise the strength of these affinity interactions. The purification factor achievable using affinity precipitation will also be impacted by the choice of AML and the operating conditions used due to the possibility of non-specific binding of impurities to the polymer backbone, which should be minimised in order to optimise the purification performance of the process. The second part of the process development would concern the optimisation of the precipitation process. The overall process of precipitation will be determined by the choice of the AML. However the vital issue is to ensure that the optimal conditions for AML precipitation are compatible with the optimal conditions for product binding. If this is not the case then a change in the process, or a trade-off possibly between purification factor and yield, must be considered.

Technological Developments

Secondary effect affinity precipitation shows some potential for use as an effective large scale purification step. It can provide high process yields and purification factors and also has been shown to be a relatively robust separation technique.[40] However several factors have limited its use at a preparative scale. One factor is the relatively low affinity of the AMLs to the product protein. Coupling of an affinity ligand to a polymer can result in up to a 1000 fold reduction in its affinity for a particular target molecule. This is a problem considering the fact that it has been thought that since affinity precipitation is a one step process, in order to achieve efficient protein purification, affinity constants should be at least one order of magnitude higher than those seen in affinity chromatography. Also the current methodology of incorporating the affinity ligands into the polymer is such that they are integrated statistically all over the polymer backbone. The resultant heterogenous nature of the AMLs can also

have an effect upon the precipitation behaviour, with different molecules responding to different levels of intensities of response stimuli which in the worse case lead to fractionation of the AML population.[43]

Oligomeric AMLs It has been suggested that well defined oligomeric AMLs may be used instead of true polymers, in order to overcome the issues detailed previously. Oligomers are made up of a finite number of monomer units and as such are smaller than polymer molecules (MW<5000g/mol). Coupling of affinity ligands to oligomers can be limited to either terminus of the molecule and it has been observed that ligands attached in this way retain their full affinity.[43] Oligomeric AMLs therefore show much lower levels of heterogeneity, making their behaviour more predictable.

Thermosensitive AMLs - Thermosenstive AMLs precipitate in response to changes in temperature, most commonly an increase in temperature past what is known as the critical solution temperature (CST). The reason for this is because the polymers used to form AMLs normally contain a mixture of hydrophilic and hydrophobic sites. At elevated temperatures (greater than the CST), the H-bridges are weakened and hydrophobic interactions between polymer molecules begin to occur causing aggregation and precipitation.[40, 44] A number of poly-N-alkylacrylamides such as poly-N-isopropylacrylamide (polyNIPAM) and poly-N,N-diethylacrylamide possess this property. Thermosensitive AMLs are an attractive option since the temperature of the affinity precipitation reaction can, in principle, be easy to control. They have been effectively used to purify proteins as well as DNA plasmids.[44, 45, 46]

pH Sensitive AMLs - pH sensitive AMLs precipitant upon usually a reduction in the environmental pH. The general mechanisms of precipitation is that upon a reduction in pH, the acidic residues present on the AML polymer backbone become neutralised and the AML molecules become hydrophobic. As a result hydrophobic interactions between the AMLs begin to occur and the molecules aggregate causing them to come out of solution.[47, 42] The commercially available high-molar-mass copolymer of methacrylic acid and methyl methacrylate, with a trade name of Eudragit S-100 is a good example of a pH responsive AML, and has been used successfully in a number of studies.[47]

Potential Applications

Affinity precipitation has to date been mainly been demonstrably used to purify proteins since the mechanisms of affinity between proteins is well understood. Affinity precipitation is a bulk separation process, however unlike ATPE, it is unable to process crude feed stocks containing solids since the initial capture step involves precipitating the product into the solid phase. Thus if the feed contained cells and cell debris, there would be no way of separating the product from such impurities. Affinity precipitation is therefore limited to be used to process clarified feed stocks. Often a dialysis step is placed before the precipitation step in order to remove process stream components which may disrupt the affinity interactions between the product and the AML, and also which may change the precipitation behaviour of the AML itself. Affinity precipitation has been reported to have been used to purify plasmid DNA from *E.coli* cell lysate.[48] In this study, a bacterial metalloregulatory protein MerR was used as the affinity ligand and was coupled to an elastin-like protein to form the AML. A plasmid containing the recognition sequence for the MerR protein was able to be successfully purified from a clarified *E.coli* lysate. In fact the purification performance using affinity precipitation was shown to be more effective than that achieved using alkaline lysis, with no chromosomal DNA or contaminating host cell proteins present in the final product.

Summary

Secondary effect affinity precipitation represents an attractive alternative, one-step, bioseparation technique. Its purification performance means that it could potentially be used to replace intermediate purification operations, including initial chromatography processes, however the presence of impurities in the final product stream, due to the occurrence of non-specific binding means that further polishing steps will inevitably be required. However since affinity precipitation is a bulk separation technique it can be used to process large volumes of relatively crude material, the only limitation being that the feed must be free of solids. The fact that affinity precipita-

tion can take this crude material (e.g. clarified cell lysate or cell culture supernatant) and purify the target product to a high level is its most appealing characteristic. The combination of a relatively high-throughput process with high selectivity means that affinity precipitation may be used effectively to de-bottleneck a biomanufacturing process. Also, unlike in primary effect affinity precipitation, the product binding and precipitation processes are uncoupled, meaning that secondary effect affinity precipitation processes are much more flexible bringing about the possibility of generating generic affinity precipitation processes, using a family of AMLs with a common polymeric backbone but different affinity ligands for specified targets. The current limitations on the process are the AMLs which are used have lower affinity constants for their product than would be desirable. Entrapment of impurities in the gel like precipitates is also another problem, generating the need for time consuming, repeated precipitate wash cycles. However technological advances in the development of oligomeric AMLs may solve such limitations. At a preparative scale, a big issue is effective phase separation. Centrifugation is a possibility, however the high shear environment can result in significant losses in yield. Filtration represents the most viable option, however further work is required in the optimisation of such processes.

3.2.3 Three Phase Partitioning

Introduction

Tertiary Butanol (t-butanol) is normally completely miscible with water. However if a sufficient amount of a salt, such as ammonium sulphate is added to the t-butanol solution, a two phase system is formed consisting of a lower aqueous phase and an upper organic t-butanol phase. If a protein is introduced to this system then through controlling the concentration of ammonium sulphate in the aqueous phase, it is possible to cause the protein to form a third interfacial precipitate phase between the upper t-butanol phase and the aqueous lower phase. This is the principle behind the technique known as three phase partitioning (TPP), which can be used to purify proteins from a crude process feed stock, the basic premise being to develop the TPP

system so that the target species of interest will precipitate out in the inter-phase whilst impurities such as lipids and other hydrophobic species will move into the organic phase, and any remaining proteins and cell debris will move into the aqueous salt phase. The precipitate can then be recovered and resolubilised in order to allow effective product capture and concentration.

The operation of a TPP process basically involves addition of a salt (usually ammonium sulphate) at a concentration lower than that required to cause "salting out" of protein to feed material. This is followed by the addition of an organic solvent. The combination of ammonium sulphate and t-butanol causes protein to precipitate out of solution with some of the t-butanol adhering to the protein molecules, leading to the formation of an interfacial precipitate between the lower aqueous and upper organic layers. Thus the interfacial precipitate is actually a protein-t-butanol co-precipitate, which floats above aqueous layer because the bound t-butanol increases its buoyancy. The exact physiochemical basis of separation is not clear but is believed to be a combination of kosmotropic action, conventional salting out effects, conformational tightening and protein hydration shifts.

Kosmotropic Action and The "Salting Out" Effect - Kosmotropic solutes are those which are said to give water structure. When such solutes are introduced to water, they become strongly hydrated, with small or negative entropies of hydration, creating a local order amongst the water molecules and also a higher local density. This interaction is known as kosmotropic action.[49] Sulphate (SO_4^{2-}) ions are one of the leading kosmotropes according to the Hofmeister series. The charge on these relatively small anions show an electrostatic interaction with the dipole moments on surrounding water molecules, causing the water molecules to form a tight, well ordered structure around the central sulphate ion. SO_4^{2-} is thus known as a strong anionic "water structure promoter". Non-ionic molecules can also be kosmotropes, with the electrostatic interactions displayed by ionic kosmotropes replaced by strong hydrogen bonding. As such in three phase partitioning, t-butanol is thought to exert some kosmotropic action. As stated previously kosmotropes are able to become strongly hydrated generating a dense structure of water molecules around a central

kosmotropic molecule. The well structured nature of the water, will mean that fewer water molecules will be available to hydrate the surface of any proteins which may be in solution. Thus if the kosmotropic solute is present at a sufficient concentration, there will not be enough free water molecules to maintain the protein in solution leading to salting out of the protein.

Conformational Tightening - It is thought that the SO_4^{2-} ions present in a TPP system further contribute to the precipitation of protein molecules by causing conformational tightening. It has been shown that SO_4^{2-} ions, carrying a divalent negative charge can bind to cationic site on the protein molecule and causing major conformational shrinkage.[49] The protein molecule essentially contracts. This in turn may force any water molecules present, away from the protein molecule and as a result may contribute to the salting out effect. Thus the precipitates formed during TPP, are not simply protein-t-butanol complexes, but may also contain sulphate ions bound to the protein surface. This effect is also thought to give sulphate ions their ability to stabilise proteins and prevent denaturisation.

Protein Hydration Shifts - Protein hydration is characterised by the well ordered structure of water molecules near the protein surface. The addition of kosmotropes may cause a change in this hydration pattern, revealing particularly hydrophobic sites on the protein surface. Interactions between hydrophobic residues of nearby protein molecules may then lead to aggregation and precipitation of the protein.

t-butanol Flotation - One mechanism in TPP which has not been thoroughly investigated and as such is not well described is the role of t-butanol causing the precipitate to float on top of the aqueous layer in the form of the interfacial precipitate.[49] t-butanol is known to be a kosmotrope and is also thought to be a crowding agent, which both contribute to reducing the solubility of the protein and the formation of the precipitate. However t-butanol is also thought to stick to and bind some proteins through interactions with non-polar sites on the protein surface. t-butanol has a relatively large specific volume, and a result a low effective density. Thus when the protein precipitate forms in TPP (which is actually a protein-sulphate ion-t-butanol complex), the presence of t-butanol, which acts as a floatation agent, causes the pre-

cipitate to rise and float on top of the aqueous layer.

Synergistic Effects - In TPP, it is a combination of all of the above factors which results in the precipitation of the protein, rather than simply salting out effects caused by the addition of ammonium sulphate to the system. It is due to the synergistic effects on protein solubility that these factors have, which results in TPP normally being carried out using ammonium sulphate concentration lower than that which would normally cause salting out of a protein in just an aqueous solution.

Operation of a TPP Process

TPP is a generally straight forward operation, made up of four steps. The first step is the addition of ammonium sulphate, at a carefully controlled concentration, to the feed material. The t-butanol is then added to the system at a volumetric ratio to the feed material of approximately 1:1. The t-butanol is mixed into solution and the TPP system is then allowed to stand and settle before the precipitate recovery step is undertaken. Product recovery is achieved by resolubilisation of the precipitate in a suitable buffer, releasing the product into an aqueous environment, ready for further purification.

Figure 3.4 shows a schematic diagram of the individual steps which are usually taken during an TPP process.

TPP has been shown to be capable of handling clarified crude feedstocks, such as cell culture supernatant or clarified cell lysates.[50, 51] When such a feed is introduced to a TPP system, proteins will tend to move into the interfacial precipitate, nucleic acid based impurities will remain in the lower aqueous phase whilst hydrophobic impurities such as lipids will move into the organic t-butanol top phase.

Key Parameters for Process Development

TPP affects protein purification essentially through manipulations of protein solubility using a combination of a number of different effects. Due to the mechanisms of separation the degree of selectivity which is shown by a TPP process is very much dependent upon the relative differences in the characteristics of the protein components.

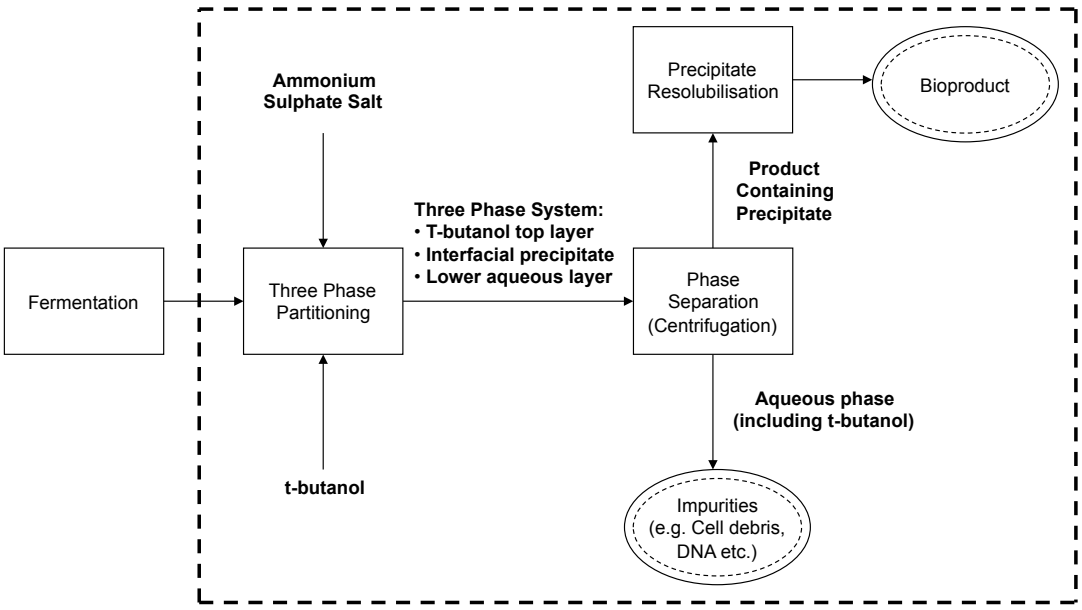


Figure 3.4: Schematic diagram showing the typical steps involved in the operation of a TPP process (The steps which actually constitute the TPP process is enclosed within the dashed lines).

Past studies have shown that certain conditions (e.g. 30% w/v ammonium sulphate, 1:1 volumetric ratio of t-butanol to feed material etc.) yield the best results, which in turn provides a good generic starting point from which to begin process optimisation.[52] With regards to critical processing parameters, it has been seen that the three factors which have the greatest impact upon the performance of a TPP process are the ammonium sulphate concentration, the amount of t-butanol which is added to the system and the system pH. The sulphate concentration required will be less than that which is required to achieve conventional salting out of proteins, but since the precipitation process relies on the synergistic effect of both sulphate and t-butanol upon protein solubility, the optimal sulphate concentration will depend on a number of factors including the amount of t-butanol present in the system and also the characteristics of the target protein (e.g. hydrophobicity, hydrophillicity, net charge for conformational tightening effects etc.)

Technological Developments

Recently affinity interactions between a product protein and an affinity ligand have been incorporated into TPP processes. In such processes the affinity ligand is found attached to a suitable polymer carrier. The affinity ligand-polymer complex is known as a Macroaffinity Ligand. During this form of TPP, it is the macroaffinity ligand-protein complex which forms the interfacial precipitate. This type of TPP is known as macroligand facilitated three phase partitioning (MLFTPP) further details of which can be found in the following section.

Scale-up Issues Associated with TPP

If a TPP process is to be run at a preparative scale then one potentially significant issue is the use of large volumes of t-butanol, especially considering the fact that the organic solvent is required in an approximately 1:1 volumetric ratio, relative to the feed material, which could be large if the TPP process is to be used to process a crude feed stock. t-butanol has a flashpoint and volatility comparable to that of ethanol, meaning that at large scale, safety may be an important factor. A specialist facility or area of the plant will most likely be required in order to house the TPP process. Such an area would require measures to be taken to minimise the risk of explosions (e.g. extractor fans, flash proof electrical components etc.). A dedicated storage facility will also be necessary to store the large volumes of t-butanol safely. Large amounts of organic solvent may also cause a waste disposal problem, or at least an increased waste disposal cost.

Potential Applications

TPP has been shown to affect purification of proteins since the mechanism of separation is directly related and controlled by changes in protein solubility (through kosmotropic action of t-butanol and sulphate ions, salting out effects, protein hydration shifts etc.). A number of studies on TPP have used crude plant extracts as the feed material[53] although the technique has also been used to purify proteins from microbial lysates.[54, 51] TPP therefore is able to process crude feedstocks, although these

feeds must be free of solids. As stated previously, the degree of selectivity shown by TPP is not high, and the level of resolution achievable will very much depend on the differences in the solubilities of the different protein components within a mixture. With this technique, since the protein is recovered in the form of a precipitate which must then be re-dissolved in a suitable buffer for further purification, it is possible to achieve extremely high concentration factors using TPP. The combination of moderate purification factors with high concentration factors makes TPP ideally suited for use as an early purification step where the aim is to not affect high purification but instead to reduce the process volume whilst maintaining high process yields. Some studies have shown that it is often necessary to perform two sequential TPP steps in order to obtain high product yields.[55] It has been found that in some cases, following an initial cycle of TPP, the majority of the product is not collected in the interfacial precipitate, but instead remains in the aqueous bottom phase. It is only after a second cycle of TPP performed on this product containing aqueous phase, that the condition of the protein is suitable to cause it to precipitate. The process performance characteristics of TPP make it an ideal step to use as an early downstream processing step, used to capture, concentrate and purify the target protein.

Summary

TPP is a bulk separation technique which is capable of capturing, concentrating and purifying a target protein from clarified crude feedstocks. TPP is a relatively high yield process and through process optimisation can be made to be relatively selective. The selectivity will however very much depend upon the characteristics of the target molecule. Regardless with its performance characteristics, TPP is ideally suited for use as an early downstream processing operation. A number of different mechanisms are thought to contribute to the overall separation mechanism utilised by TPP, and it is the synergistic action of all these mechanisms which allows protein purification to be achieved. However this also makes the performance of a TPP process unpredictable. Since a number of interacting factors play a role in causing proteins to precipitate in TPP, the exact effect of changing one of these factors,

or an operating parameter, on the process performance is difficult to predict. Past studies have shown however that a particular range of operating conditions have given the best TPP performance in the past, and as such these can be used as a basis upon which to perform further process optimisation. Process optimisation itself must be done empirically and iteratively, due to the difficulties in predicting process performance. In general it has been found that the greater the level of knowledge regarding the target protein the greater the ease of process optimisation. For example if the isoionic point of the target protein is known, then the optimal pH is known to be 2 to 4 pH units below this. Also if the concentration of ammonium sulphate required to cause "salting out" of the target protein is known, then in TPP the optimal ammonium sulphate concentration will be approximately half to three quarters of this. Once the operating parameters have been optimised, a TPP process is relatively straightforward process, and is only made up of a few different steps. Being a bulk separation technique, TPP is scalable; however several considerations must be taken into account when scaling up the process, including the consequences of using large volumes of a flammable organic solvent. Shear issues at scale may also complicate the process and these are factors which must be evaluated either at pilot scale or using some form of small scale mimic, as they may significantly alter the performance of the TPP operation. Compared to other bulk separation techniques, TPP is a relatively inexpensive operation. The equipment requirements are low, meaning a relatively modest level of capital expenditure is needed and since the technique does not utilise any type of consumables (e.g. polymers, affinity ligands etc.) the operating costs are also kept low. Some costs may however be incurred due to the handling and use of large volumes of organic solvents which may have an impact upon the cost of waste disposal and safety requirements.

3.2.4 Macroligand Facilitated Three Phase Partitioning

Introduction

One of the main limitations of TPP is its degree of selectivity which, is very much dependent upon the characteristics of the proteinaceous components present in the feed stream. In MLFTPP, the selectivity is increased by interfacing affinity-based interactions into TPP. Essentially a polymer, showing affinity for the product protein is introduced into the TPP system. This affinity polymer, termed the macroaffinity ligand, is then made to come out of solution, along with the target protein to form an interfacial precipitate. The precipitate can then be recovered, and the product is eluted from the polymer. In this way the purification factor of a TPP process can be made to increase from a figure of, for example 2-fold, up to 60-fold.[56] Polymers generally have both hydrophilic and hydrophobic sites upon their molecular surface. They can also have a net charge which can be controlled by pH. These are characteristics which are also common with proteins, and it is this similarity which forms the basis of MLFTPP. Certain polymers (e.g. Eudragit S-100), when introduced to a TPP system can be made to form an interfacial precipitate. As with proteins a combination of kosmotropic action and salting out effects are thought to cause the polymer to become insoluble and come out of solution.[56] The exact role of the macroaffinity ligand in the precipitation of the target protein in an MLFTPP system is not well understood. It is possible that the interaction between the polymer and the target protein is an additional force reducing the solubility of the target protein. For example the polymer may be acting as a crowding agent, preventing water molecules from reaching the surface of the protein reducing its solubility. It is also possible that the presence of the polymer provides additional hydrophobic sites encouraging the aggregation of protein-polymer complexes and the formation of the interfacial precipitate.[56] Regardless, proteins which have no affinity for the polymer do not collect in the interfacial precipitate, meaning that MLFTPP has a significantly higher level of selectivity than normal TPP.

Operation of a MLFTPP Process

The first step in a MLFTPP process is to introduce the macroaffinity ligand to the feed. The polymer will normally be introduced at concentrations ranging from 0.5% up to 5% w/v. The feed-polymer solution mixture is then subjected to a normal TPP process, with ammonium sulphate added followed by t-butanol. Based on the literature reviewed, ammonium sulphate salt is added directly to the aqueous feed-polymer solution, at a concentration of between 20-30% w/v. This is in line with the average amount added when using normal TPP. t-butanol is then added to the aqueous solution. Again from the literature reviewed, the average amount of t-butanol added tended to be at a volumetric ratio of aqueous solution to t-butanol of approximately 1:1, although in some cases more t-butanol was added. This mixture is then agitated and left to incubate in order to allow the three phase system to develop.

Once the interfacial precipitate has formed, the next step is to recover the product-polymer precipitate using a solid-liquid separator. Recovery of the product is then usually accomplished by resolubilising the precipitate in a suitable buffer causing the product and the polymer to dissolve and go back into an aqueous solution. The re-solubilisation buffer should be chosen to disrupt the interactions between the macroaffinity ligand and the product protein although this may be combined with the next step, involving the re-precipitation of the polymer. In order to separate the product from the macroaffinity ligand, the physicochemical environment is normally altered in order to cause precipitation of the polymer (whilst maintaining the product protein in solution). This can be done in a number of ways. From the literature reviewed, re-precipitation methods included adding salt in order to cause salting out of the polymer and altering pH to cause precipitation. Altering pH to extreme levels can also disrupt the affinity interactions between the macroaffinity ligand and the product protein.

Once the macroaffinity ligand has been re-precipitated, the next step is to separate the polymer from the product, which can then go on to further purification. The product is collected in the supernatant whilst the precipitate is collected in the form

of a dewatered compact gel. Two options then exist, in terms of further processing of the precipitate, either to wash the precipitate to recover any product which may remain bound to the macroaffinity ligands, or to recycle the polymer for reuse in the next cycle of MLFTPP. Some studies have tried to further purify the supernatant from this step by subjecting it to another cycle of normal TPP. It has been shown than using such a follow-up technique can allow the product to be recovered and purified to homogeneity, with an overall purification factor of 95-fold.[56]

Figure 3.5 shows the typical steps involved in one MLFTPP cycle.

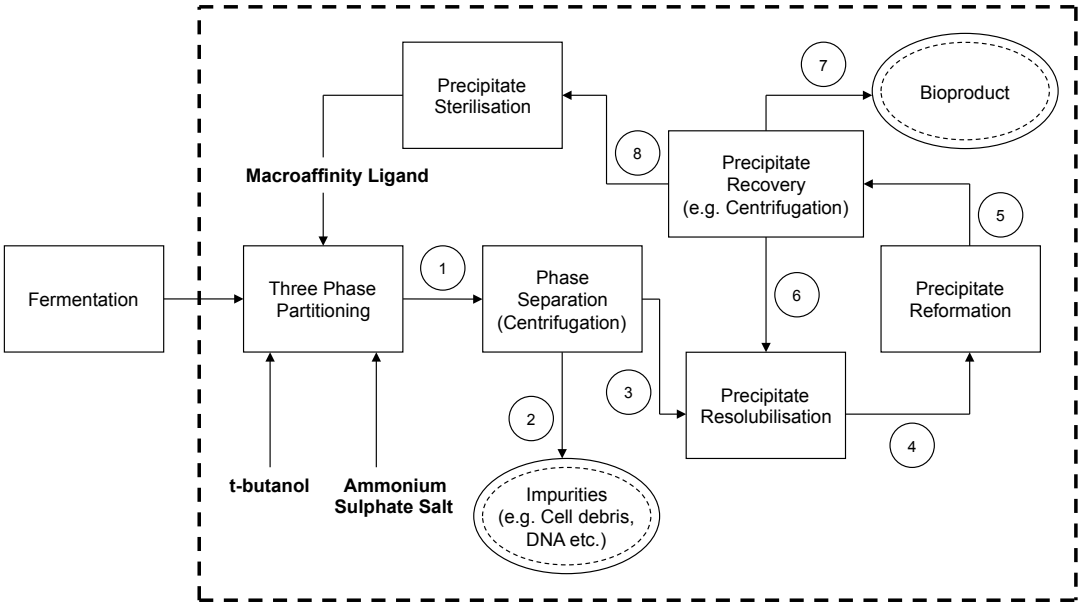


Figure 3.5: Schematic diagram showing the typical steps involved in a MLFTPP process (The steps which actually constitute the MLFTPP process is enclosed within the dashed lines)
1. Three Phase System The first step is to perform a solid-liquid phase separation (e.g. via centrifugation) in order to recover the product. *2. Supernatant* The supernatant from this first solid-liquid separation step. *3. Precipitate* The solid precipitate containing the product of interest is recovered from the solid-liquid separation step for further processing. *4. Aqueous Solution* The next step is to re-precipitate the polymer whilst maintaining the product in solution. *5. Two-phase system* The polymer is present in the form of a precipitate, whilst product remains dissolved in an aqueous solution. *6. Supernatant* The product containing supernatant from the solid-liquid separation step can be passed on for further processing. *7. Polymer Precipitate* The precipitate can be passed back for re-solubilisation. *8. Polymer Precipitate* If the precipitate can be recycled and sterilised before being used again in the next cycle of MLFTPP.

Key Parameters for Process Development

Process development of a MLFTPP process will be similar to that of a normal TPP process. The technique once again relies on manipulating the solubilities of a target molecule through the addition of ammonium sulphate and t-butanol. The critical

process parameters in MLFTPP are therefore essentially the same as those of a normal TPP process, not employing any affinity technology. Thus ammonium sulphate concentration, t-butanol concentration, the pH and the temperature will all affect the performance of the process. The mechanisms which cause polymer precipitation are thought to be the same as those which cause the precipitation of proteins using TPP. An additional process parameter now of course is the choice and amount of polymer which is added to the TPP system. This will to a certain extent depend upon the concentration of product present in the feed. The introduction of the macroaffinity ligand also has implications beyond the three phase forming step. Once the product has been captured in the interfacial precipitate it then needs to be eluted from the protein-polymer complex. Thus optimal conditions which allow for the resolubilisation of the precipitate and then selective re-precipitation of the macroaffinity ligand will need to be investigated. In this regard MLFTPP shares several aspects with affinity precipitation.

Technological Developments

MLFTPP is itself a relatively new bioseparation technique and can itself be considered a technological development and an extension of the normal TPP separation technique. MLFTPP differs from TPP in its use of an macroaffinity ligand. From the literature reviewed, specially engineered polymers, synthesised through the complexing of an affinity ligand to a polymer backbone as is done in secondary effect affinity precipitation, have not been used for MLFTPP. Instead, most examples have utilised the affinity of target proteins to the polymer molecules themselves. Polymers which have been used include alginate esters, Chitosan and Eudragit S-100.[55, 57, 58]

Potential Applications

MLFTPP, like TPP has only been shown to be effective at purifying proteins. This is because the mechanisms of separation in TPP are based on reducing the solubility of proteins. MLFTPP has also been used to sequentially precipitate different protein components of the feed by running several cycles of the technique. Thus the bot-

tom aqueous phase from the first MLFTPP cycle is subjected to another round of MLFTPP using a different macroaffinity ligand.[55]

Summary

MLFTPP has been used as a one step purification process capable of purifying a target protein from a dual component artificial protein mixture to unity.[58] More complex feeds, such a crude feedstocks cannot be purified to such an extent; however the degree of selectivity offered by MLFTPP does make it a very attractive process option. MLFTPP is also capable of processing crude feedstocks which contain solids such as cell debris. These characteristics make MLFTPP ideally suited to be used early on in the process train, where its concentrating power and selectivity can significantly reduce the purification burden placed on any downstream processing steps. In fact it has been shown that a MLFTPP process followed by a normal TPP step can purify a target protein from a solids containing (simulated) crude feedstock, to unity.[55] The use of MLFTPP however has not been shown at a large scale, and in scaling up the process, several issues may arise. As well as sharing the same issues as are associated with TPP processes, MLFTPP processes also presents process volume concerns. MLFTPP requires the addition of a number of components to the feed stream, which based on small lab scale data, can result in up to a four-fold increase in the process volume. As stated this is based on data from small scale experiments, and it may be possible during scale up and process development to reduce the volume of additions. Since the mechanisms of TPP and therefore MLFTPP are not well understood it is difficult to predict whether this will be possible. Otherwise it will be necessary to include concentrating steps prior to using MLFTPP in the process train, which may in turn negate the advantages offered by MLFTPP of processing solids containing crude feed stocks.

3.2.5 Crystallisation

Introduction

Protein crystallisation has long been used in analytical biochemistry. The growth of large protein crystals for the X-ray analysis of protein structure is a common technique and one which is well understood. However the use of protein crystallisation as a method of protein purification has not been extensively investigated. Based on the experience of protein crystallisation for structural analysis, it is generally accepted that the presence of impurities within the protein solution makes crystallisation more difficult, which rules out its use as a purification technique. However it should be considered that the needs of the protein crystallographer do not entirely match those of a biochemical process engineer.[59] For protein structure analysis, large crystals with a uniform structure are vital, however neither of these are critical outcomes when dealing with protein purification.

The basic concept behind bulk protein crystallisation is essentially to manipulate the physicochemical environment of proteins within the process stream, such as to cause the target product protein to crystallise out of solution whilst impurities remain dissolved in the process fluid. Protein crystallisation basically involves introducing some physicochemical change which will cause the protein concentration in a solution to become higher than that which would be possible without the change being implemented. When this occurs and the protein concentration of the solution is higher than should be possible at a particular set of conditions, the solution is said to be supersaturated. This is a thermodynamically unstable state for the solution to be in. If the conditions are then right, the protein may then come out of solution and crystallise, reducing the concentration of the protein remaining in solution until it reaches a more thermodynamically stable saturated state

Nucleation and Crystal Growth - With regards to protein crystallisation, there are two important kinetic parameters. These are the rate of crystal nucleation and the rate of crystal growth. Nucleation is the process whereby new crystals spontaneously form whilst crystal growth is the process whereby crystals incorporate additional

protein molecules, causing the crystal itself to increase in size. The rate at which these two processes occur is dependent upon the concentration of both the protein and the reagent which causes crystallisation.

Crystallisation Phase Diagram - The crystallising behaviour of a protein can be accurately described by a crystallisation phase diagram as shown in Figure 3.6.

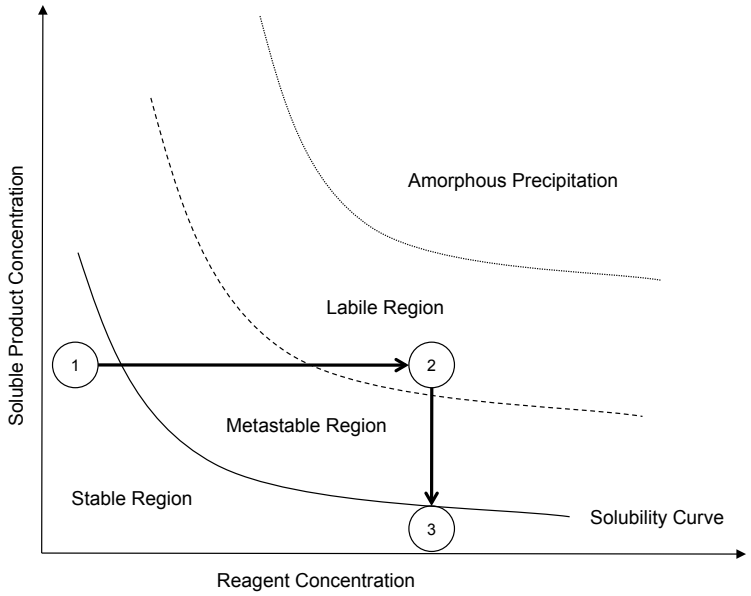


Figure 3.6: Schematic diagram of a typical crystallisation phase diagram (from Jacobsen et al., 1998)

In Figure 3.6, the concentration of the target protein is plotted against the concentration of the crystallisation causing reagent. The solid line represents the solubility curve of the system. At concentrations below this line, the protein solution is undersaturated and no crystallisation occurs. Above this line is the metastable region of the phase diagram. At these concentrations, crystal growth occurs, although the concentrations are not high enough to cause spontaneous crystal formation (nucleation). Above the dotted line is the labile region, where the concentrations of both the protein and the reagent are high enough to cause both nucleation and crystal growth. At concentrations even higher than this, above the finely dotted line, concentrations are so high that protein aggregation and precipitation occurs. The metastable region and labile region represent concentrations at which the solution is said to be

supersaturated.[59]

With a typical process stream the concentration of product will initially be below the solubility curve and as such the product protein will be present in solution. The principle behind bulk protein crystallisation is to alter the conditions of the process stream in order to move into the labile region of the phase diagram allowing crystal nucleation and growth to occur. This may be done for example by increasing the concentration of the crystallisation causing reagent (point 1 to 2 in Figure 3.6). Once in the labile region, crystals will form and grow. As this occurs, the concentration of the protein remaining in solution will drop until the protein solution becomes simply saturated, and the operating point moves back down to the solubility curve on the phase diagram (as shown by the movement from point 2 to 3 in Figure 3.6). The position at which operating conditions fall back to the solubility curve (as shown by the circle numbered 3 in Figure 3.6) will determine the yield of the process. At this point crystal growth has stopped and any product protein remaining in solution will not be incorporated into the crystals. In order to determine the potential yield from a particular crystallisation process, it will be necessary to first generate the solubility phase diagram. The shape of the solubility curve and the size of the various regions on the phase diagram is dependent upon a number of factors including temperature and pH, as well as physical parameters such as the impeller speed used to agitate the protein solution during crystallisation. The presence and amount of crystalline material in the protein solution may also affect the phase diagram.

Primary and Secondary Nucleation - Nucleation is the process of spontaneous formation of new crystal particles in solution. As stated previously, nucleation only occurs above a certain concentration threshold as shown by the dashed line in Figure 3.6.[60] In this labile region, the concentrations of both the crystallising reagent and the target protein are sufficient to cause new crystals to form. The labile region is actually further split into two sections, the primary nucleation zone and the secondary nucleation zone. In the primary nucleation zone, which exists at higher protein and reagent concentrations, new crystals form spontaneously. In the secondary nucleation zone, new crystals do form spontaneously, however crystals must actually already be

present in the protein solution in order for this to occur. Thus secondary nucleation will not occur in a crystal-free environment.

Kinetics of Crystallisation - A crystallisation process can be described by two kinetic parameters; the nucleation rate and the crystal growth rate. The nucleation rate is defined as the rate at which new crystals appear in a protein solution.[59] The crystal growth rate is defined as the change in a characteristic dimension of size, whether it be crystal length, crystal width or crystal volume, with time.[61]

Operation of a Crystallisation Process

A bulk crystallisation process is itself a relatively straightforward operation. Once the solubility curves for a particular protein with regards to a particular crystallising reagent is known, then the process of actually carrying out the protein crystallisation is relatively simple.

Crystallisation - The way in which the crystallisation process is operated is very much dependent upon the concentrations of the product protein and also the crystallising reagent which is used. Some crystallisation processes have used a salt such as ammonium sulphate to drive the protein solution into the supersaturated region of the phase diagram, whilst others have relied on the use of changes in pH.[60, 59] Regardless of which technique is used, the principle behind this stage of the process is to cause the process fluid to become supersaturated with the product protein. A choice must then be made as to whether to operate the crystallisation process initially in the labile region of the phase diagram or whether to operate it initially in the metastable region. It has been argued that operating the crystallisation process initially in the metastable region, in which nucleation does not occur, provides the best results as this allows the process engineer to control the amount, and therefore the size of the crystals which form.[60] The generation of a smaller number of crystals with larger sizes, is attractive as it will increase the ease of crystal recovery downstream. However this requires the "seeding" of the crystallisation process. Thus small crystals must be introduced into the process stream, which can then be "grown". Alternatively, the process fluid can be forced into the labile region of the phase diagram, in

which nucleation does occur. Thus seeding is not required, however this does lead to the generation of smaller crystals which increases the difficulty of crystal recovery. Regardless of the technique used, crystal formation is the most time consuming step in the entire operation potentially taking up to 24 hours.[11]

Crystal Washing - Once the crystals have been formed and the feed has returned to a saturated level, with regards to the product protein, the next step is to recover the product crystals. This can be done using any type of solid-liquid separation process. From the literature, both centrifugation and filtration have been used successfully for the recovery of the product crystals. Once the crystals have been recovered, the next step is to "wash" them in order to remove any soluble proteins which may be present in the liquid still adhering to the protein crystals. This washing is normally done by suspending the crystals in a saturated salt solution which prevents the protein crystals from dissolving.[60] The crystals may then again be recovered using centrifugation or filtration. The protein crystals may be washed in this way a number of times to ensure a high purity of the final product.

Crystal Re-Solubilisation - Once the crystals have been sufficiently washed and are recovered, the final step is to re-dissolve the protein in a suitable buffer ready for further purification.

Figure 3.7 is a schematic diagram showing the typical steps involved in a bulk protein crystallisation process.

Key Parameters for Process Development

The crystallisation phase diagram is the key to operating an effective bulk protein crystallisation process. The phase diagram will determine the conditions required to bring the solution to supersaturation, whether nucleation occurs or not and the product yields achievable. Conceivably any process conditions which can affect the phase diagram can be seen to be a critical process parameter.

Protein Concentration - The initial protein concentration of the feed will determine the ease at which the aqueous solution can be brought to supersaturation. Through the mechanisms by which crystallisation operates it can be seen that the process

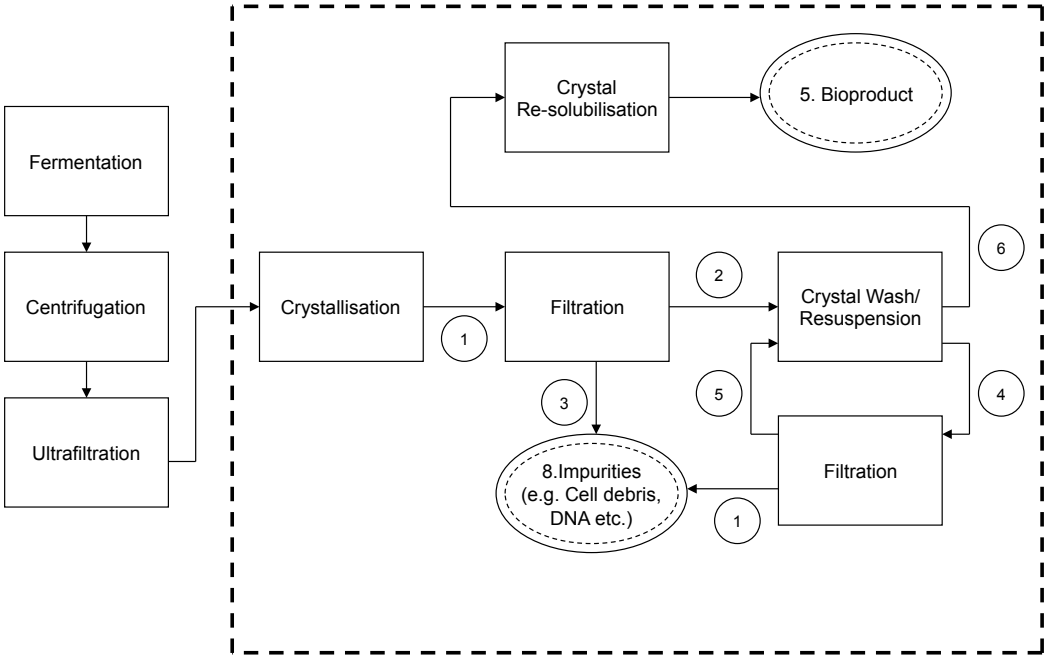


Figure 3.7: Schematic diagram showing the typical steps involved in a bulk protein crystallisation process (The steps which actually constitute the bulk crystallisation process is enclosed within the dashed lines)
1. Protein Crystals and Bulk Process Fluid Crystals need to be separated from the bulk process fluid containing impurities. *2. Retentate Protein Crystals* The product protein crystals are retained following filtration. *3. Permeate* The permeate from the ultrafiltration process will contain the bulk of impurities present in the process stream. *4. Protein Crystals and Wash Buffer* The protein crystals are resuspended in a saturated salt solution to remove any adhering liquor. *5. Retentate - Washed Protein Crystals.* Filtration should result in the recovery of pure product protein crystals. *6. Permeate Wash Buffer* The permeate will contain contaminant proteins which may have adhered to the protein surface. *7. Pure Protein Crystals* After sufficient wash cycles, the protein crystals are passed on for resolubilisation

will only work if the product protein concentration is higher than that of other proteinaceous impurities which may be present in the feed. It is for this reason that the advances in cell line technology, resulting in increased MAb product titres, have made protein crystallisation as a purification technique much more feasible.

Crystallising Reagent Concentration - If a precipitant or crystallising reagent, such as ammonium sulphate, is to be used to bring a process feed to supersaturation with regards to the target product protein, then concentration of this agent will be an important process parameter. From the phase diagram in Figure 3.6, it can be seen that if the initial protein concentration is kept constant then the reagent concentration can be used to control the region in which the crystallisation process is initiated. Thus by controlling the reagent concentration, it should theoretically be possible to determine whether nucleation occurs, or whether only crystal growth is permitted with a seeded crystallisation process being used. The number and size of crystals formed can have a significant impact on subsequent crystal recovery steps. The reagent concentration may also be used to increase the yield achievable. During crystallisation, the protein solution will drop from a state of supersaturation, back down to the maximum solubility limit and become simply saturated. The higher the solubility limit, the greater the amount of protein which will remain in solution, and thus the lower the yield of product, held in crystal form. From Figure 3.6, it can be seen that as the reagent concentration is increased, the maximum protein solubility limit tends to drop. As a result high reagent concentrations will essentially give higher process yields. Past studies have introduced the precipitant in the form of a saturated solution in order to maximise product recovery.[60]

Concentration of Impurities - The presence of proteinaceous impurities will affect the solubility of the product protein and hence the phase diagram. Protein impurities have also been shown to have a detrimental effect upon crystal growth rates.[59] It is thought that, particularly as the degree of supersaturation decreases, contaminant proteins may "blind" the protein surface, preventing further product proteins from being incorporated into the crystal lattice and hence preventing crystal growth.

pH and Temperature - Both the pH and temperature are known to have an ef-

fect upon the crystallisation phase diagram. The shape of the solubility curve and the relative sizes of the metastable and labile regions can all be affected by the temperature and pH. Thus it is important that this is considered when developing the crystallisation process.[59] It addition, pH and temperature can themselves be used as crystallising agents, much in the same way as ammonium sulphate can be used as a crystallising reagent.

Vessel Design and Vessel Operating Parameters - The design of the crystallisation vessel and the way in which it is operated (e.g. impeller speeds etc.) can have an effect upon the phase diagram. For example, high impeller speeds may result in excessive crystal breakage, leading to the development of fines or small crystal particles. This may pose a problem downstream, when it comes to recovery of the protein crystals, as the small size of the particles may make it difficult to separate them from the bulk fluid.

Nucleation and Crystal Growth Rates - As well as generating the correct conditions for protein crystallisation to occur it is also important to consider control of the crystallisation process itself. Factors such as the number of crystals which form and also the crystal size distribution, will both be determined by the rates of nucleation and growth respectively. When dealing with the first stage of a bulk protein crystallisation process, when the protein crystals themselves are actually formed, the ideal outcome is to generate a small number of large crystals. This will increase the ease of recovery of the crystals downstream. One way of ensuring that such crystals are generated, is through the use of a seeded crystallisation process, and operating within the metastable region of the phase diagram in which only crystal growth occurs, with no nucleation. Alternatively the protein crystallisation process may be operating within the labile region of the phase diagram, in which case no seeding is required. Instead, protein and precipitant concentrations at this point on the phase diagram are high enough to cause the spontaneous formation of protein crystals, or crystal nucleation. The problem here is that this may lead to the spontaneous formation of a very large number of fine crystals, which may be more difficult to recover downstream. Through process optimisation it may be possible to alter conditions in order to control the

relative rates of nucleation and growth to prevent this.

Crystal Recovery Parameters - Crystal recovery will be done via centrifugation of filtration. The operating parameters used will depend upon the characteristics of the crystals formed during the crystallisation reaction. For example if a filtration process is to be used for recovery of the protein crystals then the pore size required for the filter membranes will depend upon the size distribution of the crystals. If the crystallisation process was operated within the labile region of the phase diagram with concentrations above the primary nucleation threshold, then it is likely that the crystal size distribution will be smaller than that which would be obtained from a seeded crystallisation process, operated in the metastable region of the phase diagram.

Potential Applications

The presence of impurities within a protein solution has in the past been seen to make the generation of pure protein crystals seem impossible. However recent studies have shown that it is indeed possible to obtain pure protein crystals in the presence of impurities.[60, 59, 62] Studies have in fact shown that it may be possible to obtain protein crystals from crude feedstocks such as concentrated fermentation broths.[59] The presence of impurities in the feed has however been shown to have a detrimental effect upon crystal growth in these studies and as a result further process development will be required if the use of a bulk protein crystallisation operation early on in the process train is to be a feasible option. Indeed, an industrial scale crystallisation process has been used in the manufacture of insulin although its position within this particular process train is late on in the purification sequence where the bulk of impurities have been removed.[63] One potential issue associated with bulk protein crystallisation is process robustness, or more specifically the lack thereof. The metastable and labile regions of the phase diagram in which crystallisation processes occur are relatively small. Variations in process conditions, which may be significant if the feed is spent culture supernatant, can cause operating points to move outside of these regions and as a result prevent protein crystallisation. As a result, it is unlikely that bulk protein crystallisation could ever be used as part of a robust platform process.

Summary

Interest in protein crystallisation as a bioseparation technique was initially generated approximately a decade ago, when studies were carried out, showing that it was possible to grow product protein crystals in the presence of impurities. Protein crystallisation is inherently a highly selective process, thought to be bought about by the crystal lattice acting as a molecular "fingerprint" meaning that only the product protein will be able to be integrated into the growing crystal. This selectivity and also concentrating power makes it ideal for use as a purification process and its ability to handle impure, even crude feedstocks further increases its attractiveness. Unfortunately its widespread use has been limited for a number of reasons. Firstly whilst the crystals can be grown in impure feeds, the presence of high concentrations of impurities can have dramatic effects upon the crystal growth kinetics. "Blinding" of the crystal surface by impurities can severely inhibit crystal growth, leading to significant losses in yield. Use of feeds containing lower concentrations of impurities, by for example using the crystallisation operation later on in the process train, may allow this issue to be overcome. Another issue is the unpredictability of crystallisation processes. The efficiency of a crystallisation process is dependent upon a number of interacting factors, and it is difficult to develop any type of generic crystallisation process. As a result a large number of empirical experiments are required in order for development and optimisation. Generic approaches to process development are commercially available in the form of factorial design kits which may help reduce the amount of experimentation required and increase the speed of process development. However at the moment, this is one of the main limitations of bulk protein crystallisation. Until a deeper understanding of how different process parameters will affect the crystallisation process, it is unlikely that this particular alternative bioseparation technique will find many applications at a large scale.

3.3 Field-based Separations

Field-based separation techniques all achieve bioseparation by the imposition of some kind of force field to the component molecules within the process stream. These fields can be based on flow resistance, magnetic interactions, electrostatic interactions or affinity interactions. The techniques outlined in this section are all based on a combination of flow resistance, via the use of membranes with one of the other types of interactions listed previously. The techniques reviewed are:

- High Performance Tangential Flow Filtration (HPTFF)
- Controlled Shear Affinity Filtration (CSAF)

3.3.1 High Performance Tangential Flow Filtration

Introduction

High-performance tangential flow filtration (HPTFF) is a two dimensional separation process, separating molecules based on both size and net molecular charge. Essentially a more selective form of ultrafiltration, HPTFF can be made to utilise charged membranes to affect bioseparations, able to resolve biomolecules with the same size but different net charges. It should be noted that whilst HPTFF processes may be operated without the use of charged membranes, in this case HPTFF refers to the form in which a charged membrane is used to increase the resolution achievable.

Conventional ultrafiltration processes have been limited to only being able to separate solutes which have a ten-fold difference in size.[64] HPTFF utilises charged membranes with similar pore sizes, as those used in ultrafiltration. In this way it is theoretically possible to separate two biomolecules which have the same size but different net charges using HPTFF, overcoming the limitations placed upon ultrafiltration. The mechanisms of separation in HPTFF are the same as those seen in ultrafiltration. As a result the performance of a HPTFF process may be described using the same terms, such as flux, selectivity and throughput, as would be used for a typical ultrafiltration process.

Operation of HPTFF Processes

HPTFF is based on ultrafiltration and as such the operation of a HPTFF process is the same as that of an ultrafiltration process. Ultrafiltration rigs are usually used, with the ultrafiltration membrane replaced with a charged HPTFF membrane. Such membranes are currently commercially available (e.g. Millipore) and have been designed to be compatible with ultrafiltration equipment. Figure 6.3 shows the equipment required to run a HPTFF process.

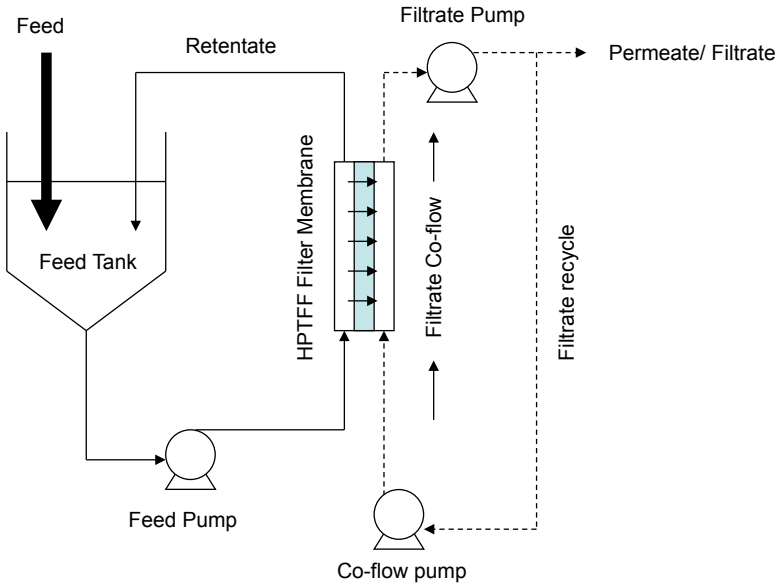


Figure 3.8: Schematic diagram of the typical equipment set-up used to run a HPTFF process

A tank holds the process feed. A pump is used to deliver material from this tank to the filter cartridge. Retained species are held in the retentate and returned to the feed tank ready for further filtration, whilst a fraction of the solvent portion of the feed along with non-retained molecules pass through into the permeate. In order to prevent the build-up of a macromolecular gel layer on the membrane surface it may be necessary to dilute the retentate with a suitable buffer, before it is pumped back over the HPTFF membrane. As detailed previously, the use of a co-current flow stream on the filtrate side of the membrane can aid in maintaining the optimal flux across the membrane

Key Parameters for Process Development

The performance of a HPTFF process will be measured based mainly on two parameters, namely the selectivity of the process and also the throughput. Obviously high values for both of these parameters would be ideal. As detailed previously, the selectivity of an ultrafiltration process and thus a HPTFF process is dependent upon the relative sieving coefficients of the different components present in the feed stream, which along with the filtrate flux rate will determine the throughput of the process. Thus there are four main performance driving parameters when dealing with HPTFF; flux rate (J), sieving coefficient (S), selectivity (Ψ) and throughput. Figure 3.9 shows the different operating conditions which can be used to affect these parameters.

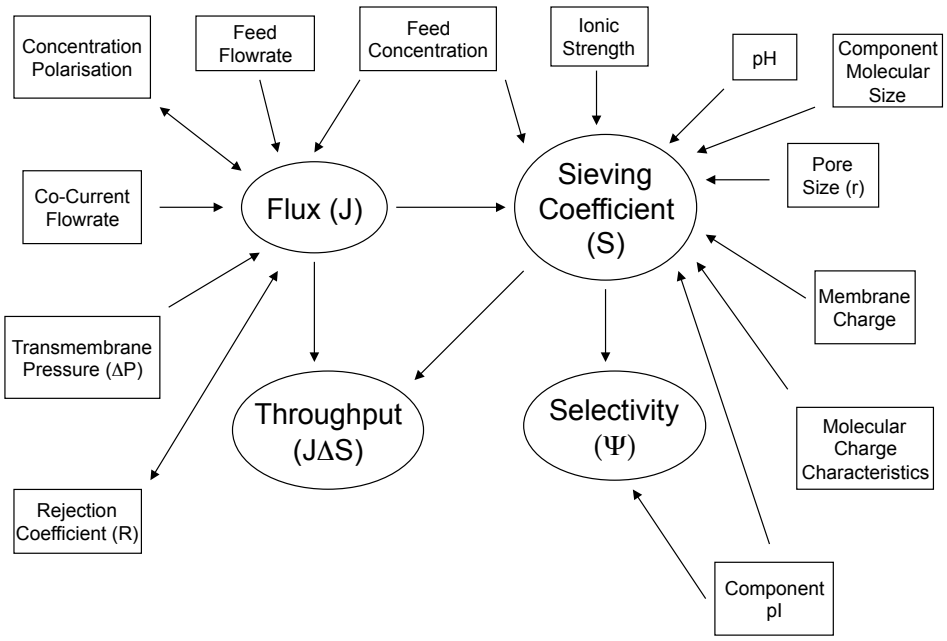


Figure 3.9: Diagram showing operating parameters which will affect the performance of a HPTFF process

pH and Component pI - HPTFF separates biomolecules based not only upon size differences but also charge differences. Thus one of the most important process parameters which need to be optimised in a HPTFF process is the pH of the feed stream. In an ideal situation, the pI of the product will be significantly different from that of impurities present in the process stream, in which case the pH can be altered

accordingly such that the product carries an opposite charge to the impurities and then can be separated using a suitably charged membrane. The size of the charge on the biomolecule can also affect the ease of separation. Biomolecules carrying a high charge will increase their effective hydrodynamic volume. As a result if the aim is to retain the product whilst impurities are allowed passage through the HPTFF membrane, altering the pH to maximise the charge density on the product and minimising that on the impurities, will increase the molecular size of the target aiding its retention whilst minimising the size of the impurities, thereby increasing the sieving coefficient and also the selectivity of the process. In such a situation it would be ideal to operate at a pH which is close to the pI of the impurities but also sufficiently different from that of the product.

Membrane Charge - The type of charge carried by the HPTFF membrane, either negative or positive, will have a large effect upon the sieving coefficient of any component within the feed stream. If the membrane is made to carry a similar charge as the product, then like-like repulsive electrostatic forces will cause product molecules to be rejected away from the membrane surface, increasing product retention and decreasing the sieving coefficient of the product. As discussed previously, the charge on the product and impurities may be manipulated through changes in the pH of the feed. The membrane charge can then be selected based on whether the product is to be retained (in which case the product and membrane should have the same charge sign), or if the product is to be allowed passage into the permeate (in which case the product and membrane should have opposite charge signs). The choice of whether to retain the product, or the impurities will be determined primarily by the relative molecular sizes of the components, which in turn can be affected by the size of the charge present on the molecules. Once it is determined which components are to be retained and which are to be allowed into the permeate the choice of membrane charge should be relatively straightforward.

Ionic Strength - The effective hydrodynamic volume of a molecule can be affected by the size of the charge present on the molecule. Molecules carrying a high charge will increase their hydrodynamic volume.[64] This effect may be utilised in a HPTFF

process in order to separate component molecules which have similar molecular sizes, but different charge characteristics. In such situations the pH may be altered such that, for example the product molecule has a high charge and thus a larger effective hydrodynamic volume whilst impurities remain neutrally charged and thus have a smaller effective volume. Appropriate membrane selection will then allow the product to be retained whilst the impurities are allowed into the permeate. The effect of changing effective hydrodynamic volume with molecular charge is greatest at low ionic strengths. At high ionic strengths the difference in hydrodynamic volume with molecular charge is not as great and as such separation through the previously described mechanism will be much more difficult. Thus HPTFF processes will be most effectively operated using low ionic strength buffers. However operating at low ionic strengths may pose some limitations as it may lead to protein denaturisation, precipitation and membrane fouling. Therefore the ionic strength should be chosen to be as low as possible whilst still ensuring protein stability. A reasonably good starting point for process optimisation has been found to be, an ionic strength of approximately 10mM.[65]

Feed Flowrate - The feed flow rate used will have an impact upon the flux through the HPTFF membrane. HPTFF operates in a tangential flow mode, with the fluid flow across the surface of the membrane used to prevent membrane fouling. High feed flowrates will therefore reduce membrane fouling which will in turn increase membrane flux. However as stated previously, concentration polarisation which occurs when molecules build up at the membrane surface can also increase flux through the membrane. Excessively high tangential flowrates may thus reduce the level of concentration polarisation which occurs and reduce the flux also. As a result an optimal feed flowrate will exist for any HPTFF system.

Transmembrane Pressure - Studies have shown that ultrafiltration processes are most effectively operated within the pressure dependent flux regime, meaning that the flux through the membrane is directly related to the transmembrane pressure. High transmembrane pressures will obviously increase flux which in turn will increase process throughput. However excessively high pressures may have several detrimental

effects, such as shear related damage to component molecules and also increased concentration polarisation. Another aspect of transmembrane pressure parameter is its uniformity during operation. Typically in ultrafiltration processes, a transmembrane pressure gradient exists across the surface of the membrane. Thus whilst a single value for transmembrane pressure is normally quoted, this value is actually only an average of the pressure exerted across the membrane. The pressure difference at the feed inlet is greater than at the retentate outlet. As a result, only a certain proportion of the ultrafiltration membrane is actually being operated at the optimal transmembrane pressure. In HPTFF, this issue is resolved through the use of a co-current flow on the filtrate side of the ultrafiltration membrane. This co-current flow exerts a reverse pressure on the membrane. Control of the co-current flowrate should allow the transmembrane pressure to be balanced across the membrane surface, ensuring that the entire membrane is being operated at an optimal transmembrane pressure.

Membrane Pore Size - The membrane pore size will obviously have an effect upon the sieving coefficient of the different components within the feed solution. Molecules which are too large to penetrate the pores will obviously be retained by the membrane whilst those which are small enough to pass through will be allowed passage into the permeate. The size of the pores of the membrane will obviously need to be carefully selected in order to maximise the differences in the sieving coefficients of the product and the impurities. Such selection will also need to account for changes in the effective hydrodynamic volume of the component molecules, with operating pH and also the charge which is present on the product, the impurities and the HPTFF membrane.

Technological Developments

HPTFF can itself be considered a technological development of ultrafiltration. The use of a charged membrane allows molecules to be separated based not only upon their relative sizes but also the electrostatic charge which they carry. Appropriate configuration of the operating pH should therefore allow, similarly sized molecules with different charge characteristics to be separated. Past studies on HPTFF have been carried out using commercially available polyethersulfone, ultrafiltration mem-

branes which have been chemically modified to carry an electrostatic charge. However it has been argued that since ultrafiltration membranes were never intended for high resolution purification, the relatively large pore size distribution observed with these membranes may be limiting the ability to exploit effectively the differences seen in variations in hydrodynamic volume with molecular charge, thereby reducing the resolving power of the technique.[66] Indeed modelling of a HPTFF process has shown that significant improvements in purification can be achieved through a narrowing of the membrane pore size distribution. A similar improvement in performance can be achieved by increasing the charge density on the membrane surface, as this will increase the size of the attractive/ repulsive electrostatic interactions occurring with the molecular species within the feed. Obviously HPTFF is still an emerging technology, however there are many examples of it being adopted and as its use becomes more widespread, membrane manufacturers and suppliers will likely find a market for specialised HPTFF membranes which have been specifically designed in order to turn ultrafiltration into a high resolution purification step.

Potential Applications

Being a membrane based operation; HPTFF can obviously be used to purify any type of target molecule, whether it is a recombinant protein, a monoclonal antibody or plasmid DNA. However its true power is in its ability to the resolve molecules of the same size. Thus whilst HPTFF can separate plasmid DNA from host cell proteins, this type of separation would be possible using normal ultrafiltration since there is a large difference between the size of a plasmid and that of a protein molecule. HPTFF can be used to separate proteins which are of the same size but have different charge characteristics and it is for this reason that the purification power of HPTFF can only be fully appreciated when dealing with protein separations. Indeed a recent study showed that it is possible to use HPTFF to separate two protein variants which differ only at a single amino acid residue.[67] This high resolving power coupled with the fact that HPTFF is essentially an advanced form of ultrafiltration (meaning that the necessary industrial infrastructure required for implementation is already

in place), makes the technique a very attractive high resolution protein purification "alternative". Unlike the majority of the bulk separation techniques which have been discussed, HPTFF is unable to process crude feedstocks, as the presence of solids will cause excessive membrane fouling. As a result, HPTFF is most effectively used as an intermediate purification step, using clarified feeds. Pre-processing of the feed may also be required in order to alter the ionic strength to a suitable level. Due to its relatively high selectivity, HPTFF may be used in place of the first chromatography step present in the downstream process. Its high throughput nature may then serve as a way of de-bottlenecking a downstream processing train.

Summary

HPTFF is a highly promising emerging technology. Its mechanism of operation allows molecules to be separated based both on their size as well as their charge characteristics. Unlike the bulk separation techniques detailed previously, HPTFF suffers from the same drawback as all filtration processes in that it is not able to handle crude feedstocks. The presence of solids and other large impurities will lead to extreme membrane fouling, and given the potential cost of their specialised membranes, this is clearly undesirable. Instead HPTFF is most suitable for use as an intermediate to late stage, high resolution purification step. It could be used in place of the first chromatography step, which would take full advantage of its relatively high throughput nature (compared to chromatography). HPTFF is also most suitably used for protein separations, where the resolution of a single product protein from a multi-protein mixture is the goal. The effectiveness of a HPTFF process will be highly dependent upon the complexity of the feed. The technique does not make use of affinity interactions and as such relies on there being differences between the electrostatic charge characteristics of the feed components at a particular operating pH. In reality a pH at which the charge characteristics of the product and impurities are at completely different ends of the spectrum is unlikely to exist. In such a case it will be necessary to alter the pH of the diafiltration buffer (HPTFF is normally run in a diafiltration mode) as the operation proceeds in order to resolve the protein mixture. HPTFF

is therefore an attractive bioseparation "alternative" and depending upon the charge characteristics of the feed components, can be used to achieve very high levels of purification. As it stands however, the high resolution potential of HPTFF has yet to be realised. The complexities of feedstocks encountered in actual industrial processes means that HPTFF is still limited to being a more selective form of ultrafiltration. Whilst resolution of complex feeds should be theoretically possible using HPTFF, the lack of established process development techniques and guidelines has limited the technique's use during later stages of downstream processes. Due to its connection with ultrafiltration HPTFF has been adopted in some industrial capacity, possibly more so than other "alternative" bioseparation techniques. The existence of an established industrial infrastructure for its implementation, along with the industrial familiarity with ultrafiltration technology has probably been the reason for this. Advances in membrane technology, such as the development of higher quality membranes along with more established process development techniques may eventually allow HPTFF to be used to its full potential.

3.3.2 Controlled Shear Affinity Filtration

Introduction

Animal cell cultures are often used for the production of naturally glycosylated therapeutic proteins such as monoclonal antibodies. Generally the product of interest from such cultures are secreted into the bulk process fluid and so the primary step in the associated downstream process will usually be some form of cell-separation operation before it is loaded onto the pre-requisite column chromatography steps. In order to improve the efficiency of downstream processes, engineers have begun investigating the possibility of integrating the cell culture process, with a cell separation step whilst also incorporating some form of product purification. Controlled Shear Affinity Filtration (CSAF) is one of the solutions which have recently been developed. Integration of cell culture with cell separation is not a new concept, and the use of perfusion cultures is fairly widespread. However the aim of such integrated processes

is to simply retain the animal cells in culture whilst removing the product containing culture supernatant. Subsequent downstream processing steps will then be used in order to capture, concentrate and purify the product from this supernatant. CSAF has been designed to allow simultaneous cell separation and product purification.[68]

The use of membrane systems to affect cell separations is not a new concept. Cross flow micro-filtration is an easily scalable process which has often been used in the past to obtain cell free harvests. Such processes do suffer from one drawback and that is the coupling of the cross flow shear and the transmembrane pressure generation. In cross flow micro-filtration, the shear forces generated by the tangential fluid flow across the surface of the membrane prevents concentration polarisation and hence membrane fouling. However the high fluid flow rates used to generate this shear, can cause a significant pressure drop in the feed/ retentate flow channel (as shown in Figure 3.10), which can then lead to non-homogenous filtrate flow. The result of this is non-optimal use of the membrane area and also more rapid membrane fouling.[68] Additionally if adsorptive membranes are used, the dynamic binding capacity can be significantly reduced by such hydrodynamic behaviour. The advantage CSAF has is that it effectively decouples the shear force exerted across the surface of the membrane and the pressure generation across the membrane itself.[68, 69] Thus it is possible to optimise conditions in order to minimise membrane fouling and also maximise membrane area utilisation and permeate flux.

The use of affinity membranes in CSAF imbues the technique with a certain degree of selectivity which is not seen in cross flow filtration. In this sense, CSAF is similar to expanded bed adsorption chromatography, in that it too is able to effect product purification from a crude feedstock. CSAF has an advantage over EBA in that it is capable of achieving complete clarification of the product stream, something which is generally not achievable using EBA, due to cells and cell debris being able to attach themselves, particularly with ion exchange matrices, to resin particles. CSAF has also been shown to be capable of complete integration into a cell culture process.[70] As a result there is potential for CSAF to be used in perfusion culture systems in which the product can be continuously removed from the culture supernatant whilst cells

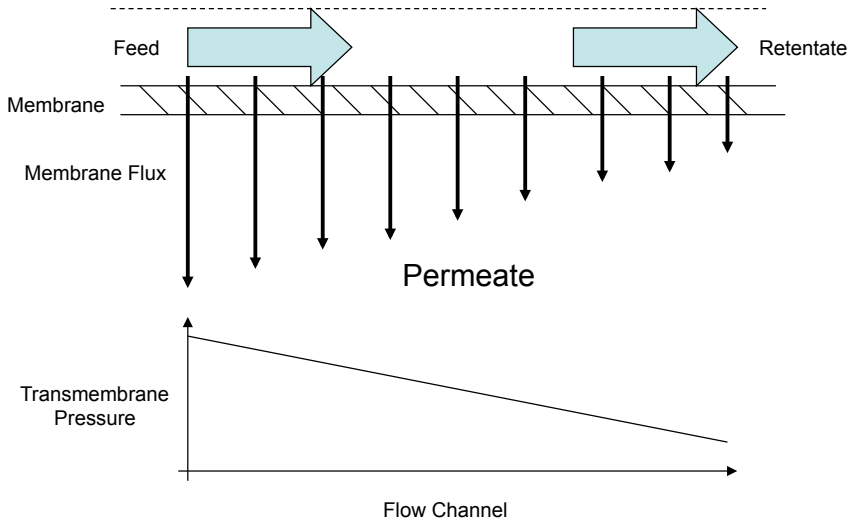


Figure 3.10: Schematic diagram showing effect of cross flow on homogeneity of membrane flux

can be retained in a viable state.

The CSAF system is essentially made up of a rotating smooth conical rotor positioned over a stack of affinity membranes (as shown in Figure 3.11). The feed is pumped into the space between the rotating cone and the membrane stack. Flux through the membrane stack can be controlled by adjusting the feed flow rate and hence the transmembrane pressure, whilst the tangential shear forces required to prevent concentration polarisation and membrane fouling is generated and controlled by the speed of the rotating cone. The basic principle is that the affinity membrane will retain cells in the retentate which can either go to waste, in a batch harvest mode of operation, or if CSAF is being used as part of a perfusion culture system, then the retentate can be recirculated back to the bioreactor. The conical shape of the rotor has been chosen as then helps to cause cells within the feed to experience a significant hydrodynamic lift away from the membrane surface. Cell culture supernatant can meanwhile penetrate the membrane stack which will contain ligands that show a specific affinity for the product. These can be used to capture the product from the filtering supernatant, whilst impurities which show no affinity will pass through into the permeate. The product can then be eluted from the membrane stack using a suitable buffer. The configuration of the CSAF system means that the binding

capacity of the membrane stack can be increased by inserting additional membranes into the stack.

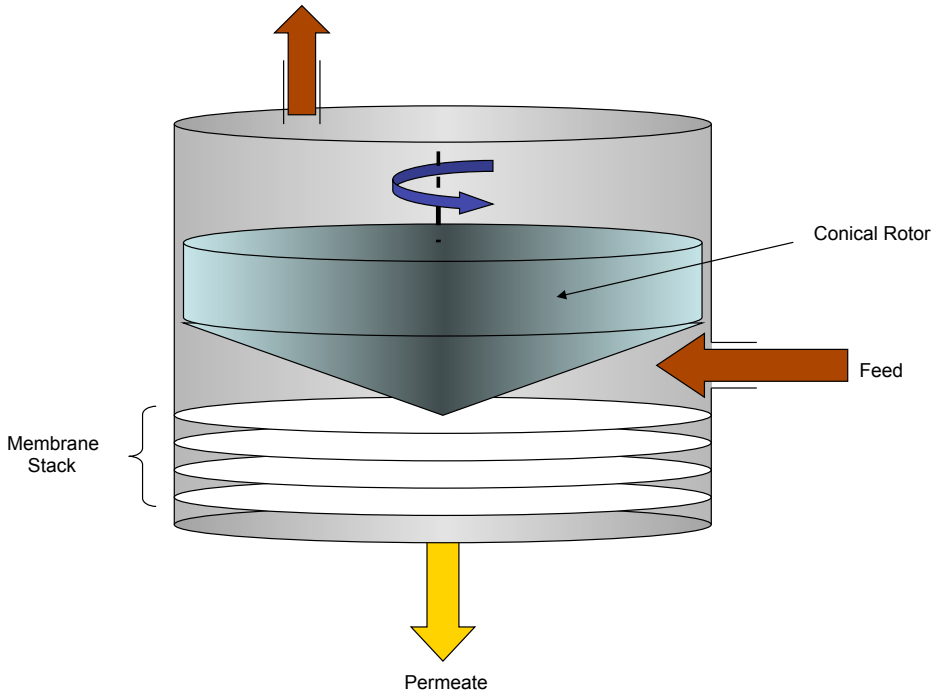


Figure 3.11: Schematic diagram showing the basic components of a CSAF system

In order to effectively implement a CSAF process in a mammalian cell culture process, there is an absolute need for robust low molecular weight ligands which have a suitable specific affinity for the product. This is of course not a trivial matter. The affinity membranes used will be directly linked to a cell culture process, and if it is to be used as part of a perfusion system, the ability to ensure system sterility becomes key. Membranes and the associated affinity ligands therefore need to be steam sterilisable or autoclavable. This therefore rules out the use of Protein A as the affinity ligand, which would most likely be the ligand of choice for monoclonal antibodies. Along with the requirement for stability under steam sterilisation, the affinity ligand must also show a high affinity for the product, whilst having a low affinity for any other components within the feed stream, and must also show low levels of non-specific binding to said components.

Operation of CSAF Processes

The use of CSAF has only so far been demonstrated at a lab scale. Once the membranes have been suitable equilibrated, cell culture is taken directly from the bioreactor or culture vessel and introduced directly to the CSAF device as described previously. Cells are retained by the membrane and can be sent either to waste, or re-circulated back into the culture vessel. The product and impurities along with a fraction of the culture supernatant will penetrate the affinity membrane stack, with impurities showing no affinity to the membrane passing straight through into the permeate. The product will meanwhile be captured by the affinity ligands on the membrane. Once the cell culture has been sufficiently recycled such that the binding capacity of the membrane stack is exhausted, the next step, again drawing parallels with affinity chromatography, is to wash the membrane stack using equilibration buffer to remove any loosely bound components. The final step is then to elute the product from the membrane stack. 0.1M NaOH may then be used to regenerate the affinity membranes. This whole process represents one cycle of the CSAF process. The number of cycles required to harvest completely one batch of cell culture will depend upon the concentration of product in the culture supernatant and also the dynamic binding capacity of the membrane stack. This procedure is also based on using the CSAF process as a batch harvest operation. As stated previously it should be possible to use CSAF as a cell retention step in a perfusion culture system. In order to do this however two independent CSAF systems will be required set up in a tandem configuration such that at any point during the process, one system will be undergoing loading whilst the other undergoes washing, elution and regeneration.

Key Parameters for Process Development

Ligand Affinity - Key to the selective nature of the CSAF process is the availability of a suitable affinity ligand. The ligand will need to display a high level of specific affinity to the product, whilst having a low affinity for impurities present in the feed stream. The ligand should also show a low level of non-specific binding to impurities

and it must be able to meet all of these requirements under cell culture conditions of temperature, pH and ionic strength. Along with these needs, the affinity ligand should stable under conditions encountered during steam sterilisation and it must be possible to couple the ligand to an appropriately selected microfiltration membrane.

Rotor Speed and Associated Shear - The purpose of the conical rotor used in CSAF is to simultaneously reduce the level of concentration polarisation and membrane fouling which occurs, through generation of tangential shear forces, whilst also providing a hydrodynamic lift force on the whole cells present in the feed causing them to move away from the membrane stack and into the retentate stream. Obviously high shear forces would be desirable to minimise the level of membrane fouling. However excessive shear can cause damage to the mammalian cells present in the feed and in the worst case, cause cell disruption. Past studies have shown that for a particular culture, a threshold shear value exists, past which the shear forces exerted are sufficient to cause cell damage and a drop in cell viability.[70]

Residence Time - The residence time (t_r) in a CSAF operation, describes the time spent by a cell, in the shear field between the conical rotor and the membrane stack. The residence time will be controlled by the feed flow rate (V'_{cycle}) and also the size of the void volume (V_{void}) in the CSAF system, which is the space in between the cone and the membrane stack.[68, 69]

$$t_r = \frac{V_{void}}{V'_{cycle}} \tag{3.3}$$

Longer residence times will tend to reduce the threshold shear value.[70]

Frequency of Exposure - The frequency of exposure (f) describes the number of times a particular cell in culture is exposed to the shear field per unit time. The frequency of exposure will depend upon the feed flow rate (V'_{cycle}) and also the total working volume (V_{total}).

$$f = \frac{V'_{cycle}}{V_{total}} \tag{3.4}$$

As is the case with the residence time, the threshold shear value will tend to

decrease with increasing frequency of exposure. High cycle flow rates will increase membrane flux, but will also increase the frequency of exposure of cells to the shear field.

Cell Status - The culture conditions will have an impact upon the threshold shear value. Cells growing in optimal conditions (ie. during exponential cell growth phase) will have higher threshold shear values than cells growing in non-optimal conditions (i.e. during early stage lag and late stage stationary/ early stage death phases).

Feed Flow Rate and Pressure Generation - The feed flow rate will influence the transmembrane pressure generated and hence the membrane flux. High membrane fluxes will obviously be desired as this will increase process throughput and thus process efficiency. However the use of excessively high feed flow rates will have deleterious effects upon the dynamic binding capacity of the affinity membrane stack as well as increasing the frequency of exposure of the cells present in culture, to the shear field within CSAF system.

Potential Applications

CSAF has been specifically designed for use as an interface operation between upstream and downstream processing steps of a mammalian cell culture. Its position within the process train will always be directly following cell culture, with crude culture being the feed material. The main selling point of CSAF is that it capable of combining clarification, product capture, product concentration and product purification into a single step, increasing the overall process yield and efficiency. A CSAF process can be operated in one of two possible modes of operation. Firstly it can be used simply as a batch harvesting technique. Once cell growth is complete, the cell culture can simply be passed into the CSAF device and loaded onto the membrane stack. Several cycles of equilibration, loading, washing, elution and regeneration may be needed in order to recover all of the product biomolecule, from the cell culture material. Alternatively CSAF can be used as part of a continuous perfusion cell culture system, in which case two separate CSAF systems (which could each be made up of a stack of filter cells) will be required such that at any one point in time during

the perfusion culture process, one CSAF system will be undergoing loading whilst the other undergoes washing, elution and regeneration steps. Perfusion systems have been successfully implemented in industrial practice, using hollow fibre membranes for cell retention. CSAF has an advantage over such systems as not only does it allow for cell retention but also allows for product capture and purification which is not possible using hollow fibre membranes.

Summary

CSAF is an integrated downstream processing technique which allows for simultaneous process stream clarification, product capture, product concentration and product purification. The integration of multiple downstream processing functions, allows for a reduction in the number of steps required in the process train, thereby increasing the overall process efficiency. CSAF has been shown to hold several advantages over conventional cross flow filtration clarification techniques (i.e. microfiltration). Besides its selective ability, CSAF also allows for the decoupling of shear force and pressure generation. This allows these two process parameters to be optimised independently allowing for greater process performance. CSAF also has an advantage over other selective clarification techniques such as EBA since, being a membrane based operation, it is able to achieve complete clarification of the process stream, something which cannot always be accomplished using EBA. CSAF may also be used as part of a continuous perfusion culture system. Such a system would require two separate CSAF units, designed to operate in tandem. The use of CSAF would present an advantage over conventional perfusion cell retention techniques, such as the use of hollow fibre membranes, since it would allow for product purification as well as clarification. The current limitation on this technology is that its application at scales beyond the lab has yet to be demonstrated. As a result its level of attractiveness for large scale application is significantly reduced. The developers of the technology have envisioned several ways in which CSAF may be scaled up. Encouragingly, large scale operation of rotating disc filters, upon which this technology is based, has been demonstrated, indicating that scale-up should be possible. However whether such scale-up schemes

work when using crude cell culture as the feed remains to be seen. Since CSAF incorporates elements of membrane chromatography, the ability to ensure uniform filtrate flux across the membrane surface is a key factor. This may become an issue during scale-up when the membrane surface area will be increased. It may also be difficult to maintain the geometric configuration of the shear fields within the void space of the CSAF module when scaling up. Regardless, further studies will need to be performed in order to determine the technical and also economic feasibility of CSAF at large production scales.

3.4 Adsorptive Separations

Adsorptive separation techniques all utilise some form of sorbent-sorbate interactions in order to achieve bioseparation. As such the techniques outlined in this section of the report are all essentially derivatives of conventional packed bed chromatography. The techniques only differ in the form and type of stationary phase to which the chromatographic ligand is immobilised. These alternative stationary phases all imbue the chromatography technique with properties which may be seen as advantageous when compared to conventional resins. The techniques reviewed in this section are:

- Expanded Bed Adsorption Chromatography (EBA)
- Monoliths
- Membrane Chromatography (Mem Chrom)

3.4.1 Expanded Bed Adsoption Chromatography

Introduction

One of the main drawbacks of traditional packed bed chromatography is the inability of the process to handle solids containing feed stocks. As a result, feeds need to be thoroughly clarified prior to processing to remove all solids such as whole cells and cell debris. Expanded bed adsorption chromatography addresses this issue, possessing the

ability to handle solids containing feed stocks allowing for simultaneous process stream clarification, product capture and concentration. The combination of these processing steps, serves to reduce the number of unit operations required in the downstream processing train, reducing capital expenditure and also operating costs. Compared to many of the other bioseparation techniques covered in this report, EBA is a relatively mature technology, which is exemplified by the fact that there are a number of reported uses of this technology for the biomanufacturing of several different biopharmaceutical products at large scale.[71, 72, 73, 74]

An EBA chromatography system shares the same components as a traditional packed bed chromatography system. A column holds a stationary phase made up of small bead particles, on the surface of which, are ligands able to bind to components within the process feed. Operation of an EBA process also proceeds along the same lines a process cycle made up of equilibration, loading, wash, elution and regeneration stages as is normally found in traditional packed bed chromatography. EBA however differs from traditional packed chromatography in one fundamental way and that is the packed bed is made to expand during operation. During the equilibration and loading stages of an EBA process, the column feed is introduced from the bottom of the packed bed, and at a flow rate which generates sufficient fluid drag as to cause individual particles to rise up the column, causing the aforementioned expansion. The expansion of the bed creates large void spaces in between the matrix particles and it is this which endows EBA with its ability to handle solids containing feed stocks. When such a feed is introduced to an EBA column, smaller molecules, such as target proteins are able to enter the pores of the resin particles, binding to the ligands which are immobilised within. Larger particles such as whole cells and cell debris unable to enter these pores, will instead pass straight through the bed via the voidage spaces, present between the particles whilst the bed is in its expanded state. EBA will normally be operated using an IEX resin, since these resins offer the highest capacity. Since EBA is to be used at a very early stage of the process (post fermentation or cell disruption), high binding capacities are a requisite. As stated previously, the expansion of the bed is caused by the fluid drag exerted on the resin particles by

the flowing mobile phase. The fluid flow rates used should be chosen such that it exceeds the terminal velocity of the whole cells and cell debris present within the feed but not that of the resin particles. This ensures that sufficient force is provided to force large impurities through the packed bed without forcing any matrix particles out of the column. To accentuate the difference between the terminal velocities of resin particles and particulate impurities, EBA resins beads are usually modified to contain inert quartz or metal alloy cores giving them greater densities than traditional packed bed chromatography resins. This serves to increase the maximum feasible fluid flow rates which can be used, helping to increase the throughput of the EBA process. EBA resins also have variable particle sizes, with bead diameters typically ranging from 50 to 400μm (traditional packed bed chromatography resins will have size distributions ranging from only 45-90μm). The reason for this is to prevent competitive occupancy amongst the resin particles and complete fluidisation of the EBA bed. Complete bed fluidisation must be avoided as this will hinder the plug flow, which is desired, within the column, leading to decreases in dynamic binding capacity and a reduction in productivity. By having a particle size distribution within the column, when the EBA bed is expanded, the larger resin particles will occupy the lower portion of the bed whilst the smaller particles will move to the top as shown in Figure 3.12.

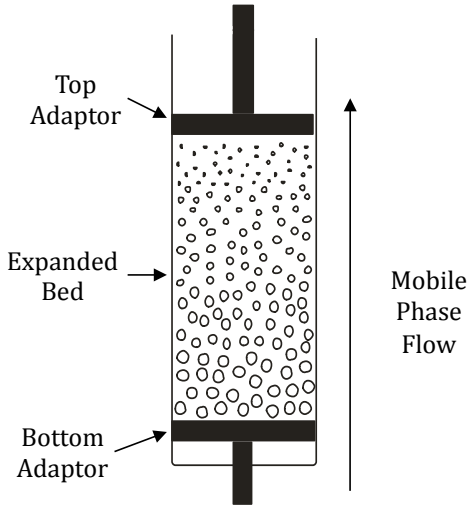


Figure 3.12: Schematic diagram showing the expansion of a packed bed in EBA and the particle size gradient generated within the column

Once the bed is expanded in this way, axial movement of resin particles is minimised and the bed does not become completely fluidised. The particle size gradient also aids in product capture. The presence of large resin particles at the bottom of the bed where the feed is introduced increases the probability of target molecules entering a pore and binding to the stationary phase. When designing an EBA process, the size of resin particles within the EBA bed must be carefully chosen. If the beads are too small then bed expansion will occur at flow rates which are comparable to the escape velocity of particulates within the feed. If the resin particles are too large, the flow rates required to cause sufficient bed expansion will be excessive to the point that residence times are sufficiently reduced to impair product binding, leading to losses in yield.

Operation of an EBA Process

During the equilibration, load and wash steps the mobile phase is pumped into the column, through the bottom adaptor at a flow rate sufficient to cause the desired level of bed expansion which is typically three times the original packed bed height. For product elution, the flow of mobile phase through the column is reversed. Equilibration buffer is initially pumped into the column through the top adaptor, which serves to collapse the bed back to it original bed height. The top adaptor is then lowered back down to the top of the bed before elution buffer is pumped through the bed at a suitable flow rate. The EBA column at this point thus becomes operated as would be a traditional packed bed chromatography system. This is done in order to reduce the buffer requirements and also to achieve concentration of the product.

Key Parameters for Process Development

Dynamic Binding Capacity The dynamic binding capacities of EBA resins will tend to be lower than those of traditional packed bed chromatography. EBA resins have a wider particle size distribution than that which is seen with traditional packed bed resins. Additionally the average particle size is larger reducing the overall surface area upon which ligands can be immobilised in order to bind product molecules. This leads

to a decrease in binding capacity. The surface area available for product binding is further reduced by the fact that EBA resin particles typically contain quartz or inert metal alloy cores, which serve to increase their density. The presence of these cores reduces the overall porosity of the particles further reducing the surface area available for binding.

Mobile Phase Flow rate The mobile phase flow rate used during operation of an EBA process is particularly important, especially during stages of the EBA cycle which involve using the bed in its expanded form. During loading several constraints upon the range of flow rates which can be used exist. A mobile phase flow rate which is lower than optimal may result in insufficient bed expansion and non-clearance of particulate contaminants (due to their terminal velocity not being exceeded), which will in turn lead to excessive fouling and prevent effective process stream clarification. If on the other hand, a mobile phase flow rate which is greater than optimal is used, this may result in a significant drop in dynamic binding capacity, leading to losses in product yield.

Mobile Phase Viscosity The expansion of the EBA bed is caused by the viscous drag exerted on the resin particles by the flow of the mobile phase around them. Higher feed viscosities will lead to greater drag forces being exerted, meaning that the mobile phase flow rate required to reach a certain level of expansion will decrease. This will place a limit on the maximum flow rate which can be used for the EBA process. High viscosity feeds may place such a limit upon the fluid flow rates which can be used that the productivity of the process may become significantly impaired. This is a particular issue since the feeds to EBA processes will tend to be fermentation broths which will have typically high viscosities. If a situation is reached whereby the viscosity of the feed is sufficiently high to impact the productivity of the process, it may become necessary to dilute the feed prior to its introduction to the EBA column.

Resin Particle Size and Distribution EBA resins will tend to have a wide particle size distribution with particle diameters typically ranging from 50 to 400μm. The exact size distribution used will have to be carefully chosen to take into account a number of different factors. For example if the particles used are too small then the

terminal velocity of the resin particles will be too close to that of the particulate impurities present in the feed stream. This will place tight limits on the suitable mobile flow rates at which the loading stage of the EBA process may be operated, which may in turn lead to a loss of process robustness. On the other hand, if the particles are too large, then the mobile phase flow rates required to cause sufficient expansion of the packed bed may become high enough that the residence time of product molecules within the EBA bed is compromised leading to reduced binding capacities and losses in product yield and productivity.

Technological Developments

The majority of developmental work on EBA has been concentrated on the generation of high efficiency adsorbents which have been designed specifically for use in EBA processes. The important parameters are the size and density of the resin particles and also the ligand and physical adsorber design.[75] The feed materials introduced to EBA processes will have typically high fluid viscosities which can lead to over expansion of the bed and in extreme cases packing of the resin into the top portion of the column. To deal with this, EBA resin particles will either be made of high density porous materials or will be synthesised by coating a high density non-porous core (e.g. quartz) with a porous material. These measures however do come with associated trade-offs. Generating high density porous resin particles can significantly increase their cost, whilst coating a non-porous core with a porous material can reduce the binding capacity of the resin. Another issue with EBA is that due to the high flowrates required to maintain bed expansion, the residence time of product molecules may become reduced which in turn can result in under-utilisation of the binding capacity of the resin. This has lead to the development of pellicular adsorbents as shown in the Figure 3.13.

These adsorbents are made up of a high density non-porous core, attached to which are thin polymeric ligands. Thus the high density core allows for the utilisation of high fluid flowrates and enables the handling of high viscosity fluids, whilst the polymeric ligands allow the resin to exhibit reduced diffusion limitations as a result

Figure 3.13: Pellicular EBA resin particle

of reduced transport lengths to the adsorbing ligands. Conventional resins require product molecules to enter into the pores of the adsorbent molecules before diffusing from the mobile phase into the stationary phase. Such protracted transport mechanisms are avoided with pellicular adsorbents. Other ways in which the capacity of the resin has been increased is through the manipulation of the chemical structure of the adsorbent layer leading to decreased mass transfer resistance as well as the design of adsorbents which actively challenge biomass-adsorbent interactions leading to decreased clogging and blocking of pores by cells and cell debris.

Potential Applications

EBA has been specifically designed for use as an early downstream processing operation, capable of performing process stream clarification, product concentration and purification thereby increasing process efficiency. To date a number of examples exist in which EBA has been used in the large scale purification of a number of proteinaceous products ranging from therapeutic monoclonal antibodies to recombinant proteins for process use such as rHSA.[76] EBA has also been shown to be compatible with a wide range of host organisms, enabling product capture and purification from microbial, mammalian and yeast culture broths. One criticism which has been levelled at EBA is its lack of robustness. Variations in the physicochemical properties of the feed material can have dramatic effects upon the performance of the process. This is mainly due to the fact that EBA is an integrated downstream processing technique which combines two traditionally independent process operations, namely

clarification and purification.[75] EBA processes will have to be developed in order to optimise both of these process and this fine balance of operating parameters may be disrupted through changes for example in the viscosity of the feed material, the solids content or indeed the product concentration. This is a particular problem considering the fact that EBA is designed for use early on in the downstream processing train, directly following fermentation for extracellular products and following homogenisation for intracellular products, where perhaps variability in the composition of the process stream is at its highest. Process robustness can be improved through greater understanding of the interplay which occurs between different process parameters, arising from the fusion of fluidised bed hydrodynamics, mechanisms of cell-surface interactions and chromatographic principles.[75] The development of such a level of understanding has been made considerably easier through recent developments in the successful modelling of EBA processes.

Summary

EBA is an integrated downstream purification technique which allows for process stream clarification, product concentration and purification. These process functions would conventionally be performed by a combination of centrifugation, microfiltration and possibly ultrafiltration processes. By combining several downstream processing operations into one step, EBA serves to increase the efficiency of the overall downstream process. Its operation is essentially analogous to conventional packed bed chromatography, however the expansion of the packed bed during loading and washing gives it an advantage over packed bed chromatography in that it is able to handle solids containing feed stocks. As a result EBA is ideally suited to being used early on in the purification process train. In terms of providing a higher throughput, cheaper processing alternative to packed bed chromatography, EBA is likely to fall short of this aim. This is due to the fact that the mechanism of separation (i.e. adsorption of product onto a suitably selected adsorbent resin) is essentially the same. Thus increases in product titre will require larger EBA beds increasing processing costs and times and decreasing process productivities. In fact, the problems which packed bed

chromatography may encounter as a result of increasing product titres will be exacerbated with EBA, as the adsorbents used tend to exhibit lower binding capacities than packed bed chromatography media. As a result of this EBA may not be considered as an alternative to packed bed chromatography, but more accurately an alternative to other less selective conventional downstream processing unit operations such as centrifugation and filtration. The introduction of selectivity at earlier stages of downstream processing can only help increase the efficiency of the process as a whole. As a result EBA may be seen as a way of decreasing the purification burden placed on subsequent downstream processing operations such as packed bed chromatography.

3.4.2 Monoliths

Introduction

In traditional packed bed chromatography, the use of compressible resins coupled with mass transfer limitations, particularly when used to capture large molecules (e.g. plasmid DNA, viruses etc.) means that limits are placed upon the feasible mobile phase flow rates which can be used during the operation of traditional packed bed chromatography processes. Such limits can therefore constrain the productivity of the packed bed process. Monoliths are a new form of chromatographic media which allow these imposed limits of productivity to be overcome. Unlike traditional packed bed chromatography resins which are made up of small porous particles, monolithic chromatography media is made up of one continuous, porous solid phase. The pores in a monolith are highly interconnected and form a pore "network" within the monolithic structure.[77] These characteristics provide the monolith with a greater level of structural stability, increasing the mobile phase flowrates at which the chromatography process may be operated at.

The pore structure of the monolith also significantly reduces resistances to mass transfer, relative to conventional packed bed chromatography, complimenting the feasibility of operation at higher mobile phase flow rates. Adsorption of product molecules to the stationary phase during packed bed chromatography requires the

product molecule to penetrate first the pores of the matrix particles via diffusion. This is a characteristically slow process and can often be rate limiting. Increasing the fluid velocities through the column can reduce the residence time and therefore binding capacity of the column. The pores found in a monolith column, meanwhile, are significantly larger than those found in conventional packed bed chromatography resin (at around 5μm as compared to pore sizes in conventional packed bed chromatography media which are in the range of 100's of Angstroms).[77, 78] The significance of these large pores is that the transport of product molecules to binding ligands is no longer facilitated by diffusional mass transfer processes but via the convective flow which is enabled by the macroporous nature of the monolith structure. As a result mass transfer of product to ligand binding sites is no longer rate limiting, allowing the performance of the chromatography process to be effectively de-coupled from the mobile phase velocities which are being used. Indeed studies on product elution profiles have shown that peak shape is not significantly affected by mobile phase flow rates, even at superficial fluid velocities in excess of 10,000cm/h.[77, 79] It should be noted that whilst the use of monolithic supports does allow for the elimination of pore diffusion, diffusion itself is not completely eliminated from the mass transfer processes which occur during separation. For example film diffusion effects must still occur for component molecules to actually move from the bulk fluid to the ligand binding sites. As a result monoliths may be thought of as possessing a certain level of "augmented diffusivity".[80, 81]

Combining the enhanced mass transfer properties along with the increase structural stability of monoliths, the limitations on fluid velocities imposed upon conventional packed bed chromatography do not apply when using monolithic media. As a result monoliths may be used to enable extremely fast chromatography processes to be operated allowing for higher process throughputs and productivity. The majority of monoliths however which have been developed display lower binding capacities (0.5 up to 12 mg/mL) than those of their conventional counterparts.[77] These lower binding capacities may be seen as a major drawback to the use of monolithic media for preparative chromatography. Whilst the increase in the feasible mobile phase ve-

locity range, may help to increase the speed for the process, this may be negated by the need for a larger number of process cycles in order to capture all of the product from the process feed. For this reason monoliths to date have been mainly used for analytical purposes.[77, 79, 82]

Monoliths are currently available in a variety of forms. Figure 3.14 shows an example of some of the different types of monoliths which are currently being used.

Figure 3.14: Examples of agarose monoliths including short columns, fibres and disks

Operation of Monolith Chromatography

From an operational perspective, monolithic columns may be used in almost exactly the same way as conventional packed beds. The only difference is that now the stationary phase of the chromatographic system is one continuous support rather that a number of individual particles. As described previously, this removes any limitations on feasible mobile phase flow rates. However the operation of the bioseparation process remains as it would be for conventional packed bed chromatography. Thus operation will still involve loading, washing, elution and regeneration, albeit these steps can now be performed in a much shorter period of time. A characteristic of monolithic columns which may be seen as an advantage is that their use eliminates the need for any sort of column packing. With regards to the manufacturing of mono-

liths, they have been synthesised using a wide range of different polymers including polymethacrylate, polyacrylamide, agarose, cellulose and silica. Active monoliths may be generated by either synthesising the polymeric monolith first and then immobilising the bioseparation facilitating ligand to it or by attaching the ligand to one of the co-monomers and then synthesising the monolith.[77, 83] The former method allows the active monolith to be generated in one step without the need for a separate immobilisation step.

Key Parameters for Process Development

As stated, the operation of a chromatography process employing monolithic media is the same as that of when utilising conventional packed bed media. As a result the process parameters which require optimisation are also analogous. The major difference is that with monolithic supports, the mobile phase velocity no longer affects the separation performance and so process productivity will be entirely dependent upon the dynamic binding capacity of the monolith.

Binding Capacity - With monoliths mass transfer of product from the bulk fluid to the ligand binding sites is facilitated by convective fluid flow. As a result mass transfer is expedited, leading to a lower dependency of binding capacity upon mobile phase velocity, unlike conventional packed bed chromatography where binding capacity and mobile phase flowrate are intrinsically linked. Instead the binding capacity of the monolith structure is primarily dependent upon the ligand concentration within the monolith structure and also the surface area available for ligand immobilisation. This is an area in which monoliths face limitations compared to conventional chromatography media. Monoliths will tend to have lower surface areas available for ligand immobilisation and thus product binding compared to their particulate based counterparts and as a result will tend to exhibit significantly lower binding capacities.

Materials of Construction - Monoliths have been constructed from a wide range of different materials, using a wide range of different synthesis methods. A book entitled "Monolithic materials: preparation, properties and applications", published in 2003 gives a good background as to the ways in which monolithic columns may be prepared.

Some of the most commonly used materials include GMA/EDMA copolymers, which are used to make the convective interaction media (CIM) monoliths as supplied by BIA separations, agarose which is commonly used for the basis of many conventional packed bed chromatography resins and silica.

Pore Structure - One factor which must be considered when utilising monoliths is the structure of the pore network. As described previously, monoliths are continuous structures, containing a network of interconnected through-pores. The exact structure of this network, such as the distance between the nodes of the network, may have an effect upon the separation performance of the monolith. The optimal characteristics for the pore networks are however currently not well understood.

Bed Volume - Current monoliths are only commercially available at volumes of up to 800ml whilst 8L prototypes are currently being made available. The relatively small monoliths which are currently available may help to explain the relatively limited level of uptake of monoliths in large scale biomanufacturing. The reason why monoliths are currently only available at small scales is due to the difficulty in synthesising large continuous supports which have a consistent pore network structure.

Technological Developments

Recent developments in monolith synthesis have lead to the generation of supports known as cryogels, capable of handling solids containing feed stocks. Cryogels are extremely porous structures prepared by cryopolymerisation at temperatures below -10^{o}C. Figure 3.15 gives a brief summary of how cryogel monoliths are prepared. The major characteristic of cryogels which separates them from standard monoliths is the presence of gigapores within the cryogel structure. Cryogels contain a system of very large interconnected pores, larger than those which would normally be found in normal monoliths. These large pores allow for the passage of large particles such as cells and cell debris which in turn allows cryogels to be used as chromatographic media for capturing product molecules from crude, solids containing feed stocks. In this way, similar to expanded bed adsorption (EBA) chromatography, cryogels may be used to introduce selectivity early on in the downstream processing train, allowing for si-

multaneous clarification, product concentration and purification. For example recent work has lead to the development of cryogels capable of direct capture of recombinant lactate dehydrogenase from crude *E.coli* fermentation broth. These cryogels were synthesised by radical cryopolymerisation of acrylamide, allyl glycidyl ether and N,N-methylenebisacrylamide, leading the generational of a continuous matrix containing a network of interconnected pores with sizes ranging from 10 to 100 μm.[84] The procedure for generating cryogels is technically straightforward, and the materials required for synthesis are relatively cheap. As a result chromatography using cryogels and the chromatographic media may be seen as a low cost alternative to EBA which can utilise quite costly stationary phases. Also due to the ways in which monoliths allow for de-coupling of mobile phase velocity from process performance, the limitations on feasible fluid flow rates used in EBA, particularly when dealing with viscous feed stocks, are not encountered when using cryogels.

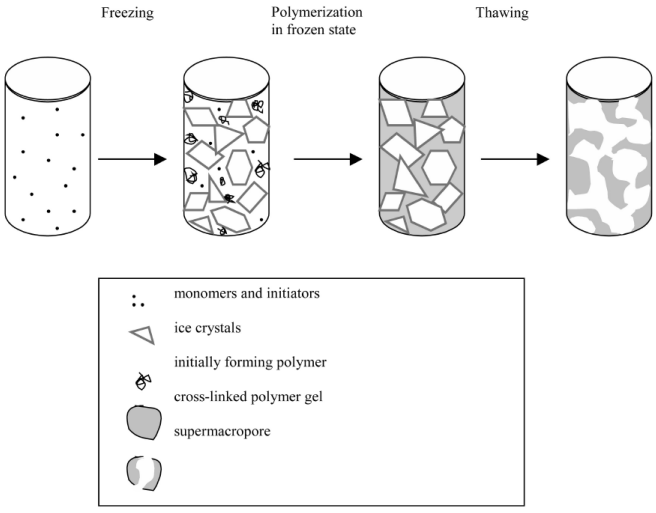


Figure 3.15: Schematic showing procedure for cryogel synthesis
Diagram taken from Jungbauer et al., 2004.[77]

The current disadvantage of cryogels however is their relatively low binding capacities. Currently, binding capacities do not exceed 1mg/mL which is siginificantly lower than that typically seen with EBA resins. However this particular type of monolithic support is a relatively new development and thus there is potential for further improvement which may lead to increased capacities.

Potential Applications

Monoliths, essentially being an alternative form of chromatographic media, may be used to affect a wide range of different bioseparations. Thus monoliths may be used in ion exchange mode, hydrophobic interaction mode or even used for affinity chromatography. One area in which monoliths have a definitive advantage over conventional packed bed media is when dealing with the purification of large molecules. Due to the relatively large pores present in monoliths, they are particularly suited for use in the separation of plasmid DNA (pDNA). Such molecules will tend to have a particle radius greater than 45nm. With conventional packed bed chromatography media, this particle size is often sufficient to ensure that that pDNA is excluded from the pores of the individual media particles and as a result conventional packed bed chromatography media will display relatively low binding capacities for pDNA. Monoliths on the other hand contain large pores, which in turn allow a greater level of exposure of bioseparation facilitating ligands to pDNA molecules in the mobile phase. Monoliths may display pDNA binding capacities of up to 12mg/mL.[85, 86] Another application which has exploited the ability of monoliths to handle relatively large particles, is the use of monoliths in the separation of viruses.[87, 88] Virus particles may have a radius in excess of 800nm. However many monoliths have sufficiently large pores as to allow the free movement of viruses within the monolith structure. This would not be possible with conventional packed bed media since, as is the case with pDNA, virus particles will tend to be much too large to penetrate the pores of the matrix beads. As a result purification of viruses is currently normally carried out using microfiltration or continuous ultracentrifugation. The use of monoliths presents a much more selective method of virus purification.

Summary

Monoliths are a relatively new form of chromatographic media which allows the limitations on mobile phase flow rates placed upon conventional packed bed chromatography to be overcome. Monoliths are essentially one continuous porous structure, containing

a network of large interconnected through pores. These characteristics allow monoliths to eliminate the slow pore diffusion step from mass transfer processes enabling rapid adsorption of product molecules to the ligands present on the stationary phase. Monoliths are currently commercially available at volumes of up to 800mL, although 8L prototypes have been made available by suppliers. The main limitation on monoliths is currently the relatively low surface area per unit volume ratio compared to conventional packed bed resins. As a result monoliths will tend to exhibit significantly lower binding capacities that their particulate based counterparts. As a result if monoliths were to be used for preparative purposes, the advantages of increased processing speed due to higher feasible flowrates may be negated by the fact that a greater number of process cycles are required. It is most likely for this reason that to date monoliths are mainly being used for analytical purposes, whereby the advantages of being able to perform ultrafast separations may be fully exploited. Recent developments have lead to the generation of cryogel monoliths, which contain gigapores capable of allowing the passage of large particulates such as cells and cell debris. This has lead to interest in the use of cryogels in place of other adsorptive based separation techniques which are capable of handling crude feeds such as EBA. These cryogels are relatively cheap and straightforward to synthesise and may be a more cost effect alternative to EBA. However current limitations in binding capacities are once again the main drawback to their utilisation. Based on this it seems that whilst monoliths do offer significant benefits over conventional chromatography media, the fact that they are only able to offer modest binding capacities means that they can only currently be considered for analytical use. However it should be borne in mind that monoliths are a relatively new technology and as such there is potential for further development. If the hurdle of binding capacity can be overcome, then monoliths may be a very attractive alternative to conventional packed bed chromatography media.

3.4.3 Membrane Chromatography

Introduction

One of the major limitations of conventional packed bed chromatography is the coupling of fluid flow and process performance. Component adsorption and de-sorption are both rate limited by diffusion facilitated mass transfer. As a result, high fluid flow rates can lead to a decreased residence time which can cause a reduction in binding capacities and hence process productivity. Membrane chromatography has been studied as an alternative to conventional packed bed chromatographic media. The benefit of these adsorptive membranes is the shorter mass transfer times associated with movement of molecules to the ligand binding sites, which rather than being located in the pores of adsorbent particles containing stagnant fluid, are present on the through-pores of the membrane allowing for convective fluid flow and faster diffusion rates to active sites. Thus in a way membrane based chromatographic media may be thought of as a form of monolithic resin.[89] A comparison of the mass transfer processes which are occurring in packed bed chromatography and that which is occurring in membrane chromatography is shown in Figure 3.16.

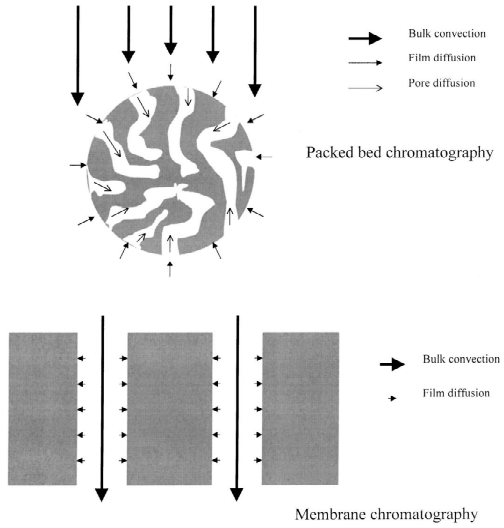


Figure 3.16: Schematic showing the mass transport phenomena occurring in conventional packed bed chromatography and membrane chromatography. (Taken from Ghosh, 2002.[89])

As can be seen, solute diffusion is still a component of the mass transfer process,

however due to the elimination of pore diffusion from the transport phenomena, the overall process of solute binding is much faster. This is reflected by the fact that membrane chromatography can be used to perform adsorptive separations in up to one tenth of the time taken by conventional packed bed chromatography.[90]

Being based on membrane separations, membrane chromatography may be operated in a number of different configurations, varying in the type of membrane used and the relative direction of fluid flow. Figure 3.17 shows some of the different system configuration which have been investigated.[89] Flat sheet membrane chromatography is the most analogous to conventional packed bed chromatography. Here flow is introduced, normal to the surface of the membrane. Issues related to the use of this mode of operation include the difficulties in ensuring uniform flow distribution across the entire surface of the membrane, particularly when considering the typically large diameter to length ratio of most membrane chromatography modules.[91] Hollow fibre membrane chromatography modules have an advantage over flat sheet membrane configurations in that they offer very high surface area to volume ratios and as such are perfectly suited for handling large process volumes. Also the cross-flow pattern across the surface of the membrane can help prevent the accumulation of particles near the pore entrance, which could potentially reduce binding capacities. This particular configuration does however have some disadvantages as high levels of product exposure near the entrance of the hollow fibre, can lead to broadening of the breakthrough curve when operating in bind and elute mode. This in turn can lead to under utilisation of the membrane adsorber and process inefficiencies. Radial flow adsorbers are claimed to be suitable for use in large scale applications, however the flow patterns in such modules are difficult to predict and control. Membrane areas increase in a radially outward direction which can lead to difficulties in maintaining superficial fluid velocities as they pass through the membrane. Despite these issues, radial flow membranes have proven popular amongst manufacturers.[92]

A review, gathering information on the reported usage of the different types of membrane configurations showed that the flat sheet configuration is by far the most popular form of membrane chromatography currently being used or investigated, pos-

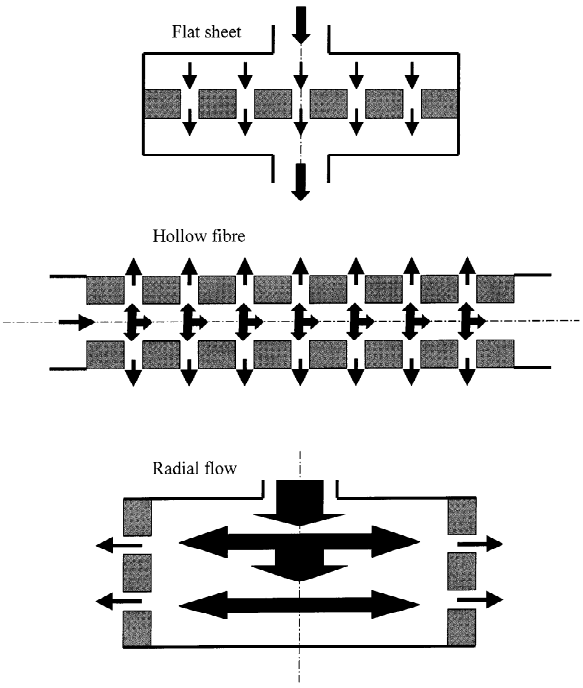


Figure 3.17: Diagram showing different flow configurations utilised in membrane chromatography. (Taken from Ghosh, 2002.[89])

sible due to the parallels between its operation and that of conventional packed bed chromatography.[89]

The membranes used for membrane chromatography are generally based on those which are used for conventional microfiltration operations rather than ultrafiltration. Since the chromatographic media in membrane chromatography is simply a membrane, the pressure drop issues associated with using high flow rates in conjunction with compressible resins in packed bed chromatography, are avoided. This further enhances the ability of membrane chromatography to offer high throughput separations.

Operation of Membrane Chromatography Processes

Despite the use of membranes as chromatographic media, the operation of membrane chromatography processes is highly analogous to that of conventional packed bed chromatography. Membranes may be utilised in flow through or bind and elute modes of operation, with the same steps used. So for example, if operating in bind and elute mode, equilibration, loading, wash, elution and regeneration steps are all used, with

the purposes of each step the same as they would be in packed bed chromatography.

Key Parameters for Process Development

As stated, operation of a membrane chromatography process is highly analogous to that of conventional packed bed chromatography. As a result the process parameters which need to be optimised and controlled are also similar. So parameters such as the binding and elution conditions all need to be monitored.

Mobile Phase Velocity - With conventional packed bed chromatography, mobile phase velocities can have profound effects upon the separation performance of the process and also the productivity and throughput. With membrane chromatography, limitations on feasible fluid flow rates can be avoided to a certain extent, as the mass transport processes are significantly expedited through the elimination of pore diffusion phenomena. However mobile phase velocity is not completed de-coupled from process performance. An important parameter in membrane chromatography is the flow distribution across the surface of the membrane. Variations in fluid flow rates can have deleterious effects upon the flow distribution which in turn may go on to impact upon the separation performance of the technique.[91] For example, with the use of radial flow membranes, high fluid flow rates may drastically alter the flow distribution within the membrane capsule leading to mixing and non-uniform composition of material actually passing through the pores of the membrane. This may lead to under utilisation of the membrane adsorber and/or poor purification performance. Similarly, flux patterns through hollow fibre membranes, which operate in a tangential flow configuration are highly dependent upon transmembrane pressure drops and fluid velocity. Variations in fluid velocity may lead to unexpected fluxes through the membrane again leading to under utilisation of the adsorber and poor purification performance.

Membrane Design and Construction - With regards to the membrane adsorbers themselves, several characteristics have been argued to be of importance.[91] Firstly, membranes must be micro-porous in order to facilitate the free interaction of solutes with separation enabling ligands. Membranes should also be hydrophilic and neu-

trally charged so as to minimise the level of non-specific binding of biomolecules to the membrane itself. In order to handle high fluid flow rates and high transmembrane pressures, thereby capitalising on the reduction in mass transfer times possible with membrane chromatography, membranes must be designed to be physically stable. Finally, membranes must also contain chemically active sites to enable bonding of ligands which can be used to effect bioseparation. This to date remains the greatest challenge in making membrane chromatography a readily accepted bioseparation technique as difficulties have been encountered in developing inexpensive generic membranes, which contain sites within the membrane matrix capable of forming bonds with a wide range of chromatographic ligands.[90]

Membrane Materials - To date several types of materials have been successfully employed for use in membrane chromatography. Cellulose is a commonly used material for the construction of membranes for filtration purposes, however commercially available cellulose membranes used for microfiltration have relatively small pores. With membrane chromatography, these pores need to not only allow the entry and passage of macromolecules but also for the attachment of ligands which themselves may also be macromolecules in the case of affinity based chromatography. The use of microfiltration cellulose membranes thus in membrane chromatography can lead to the generation of significant transmembrane pressures which can have a deleterious effect upon performance. As a result new cellulose based membranes have had to be designed incorporating high porosities and large pore sizes (1-2μm compared to 0.1-0.7μm commonly found with microfiltration membranes) to accommodate these factors.[90] Polysulphone is another commonly used material for generating microfiltration membranes and these have been successfully employed in membrane chromatography. Polysulphone has the desirable property of having high thermal and biological resistances.[90]

Inlet Flow Distribution - Flow distribution issues are not unique to membrane chromatography. Ensuring uniform flow distribution across the surface of a packed bed is paramount to ensuring efficient chromatographic performance. However with membrane chromatography flow distribution issues are of a particular importance due

to the large frontal surface area to bed height ratio. In a flat sheet configuration, when feed is introduced to the membrane adsorber, it is desirable that this feed hits all points of the membrane surface at the same time. As stated, due to the relatively large size of this surface area, this ideal situation is rarely achieved. This results in adsorbent under-utilisation and due to non-uniform flow distribution leading to distortion of the solute front, broadening of the breakthrough curve for the membrane. The problem may be further exacerbated by inefficient flow distribution through the membrane itself, as a result of pore size distributions and membrane thicknesses, as detailed below. As a result, improvements in inlet flow distribution are key if membrane chromatography is ever to become a viable alternative to conventional packed bed chromatography.[91]

Membrane Thickness - Differences in membrane thickness across a single sheet may cause similar problems as those caused by differences in pore sizes. Fluid will again preferentially move through areas of the membrane which exhibit smaller thicknesses due to the decreased pressure drop across the membrane in these parts. This will again lead to ligand under-utilisation and a decrease in membrane capacity. Fortunately modern methods of membrane synthesis has meant that the majority of commercially available membranes for chromatography are of a uniform thickness. In cases in which membrane thickness is a problem, it has been suggested that these issues of ligand under-utilisation may be overcome by stacking membranes on top of one another in order to even out the thickness distribution.

Binding Capacity - Chromatography membranes, due to reduced surface areas for ligand binding, typically display lower binding capacities per unit volume than conventional packed bed chromatography media. This situation may be further exacerbated by the issues outlined previously. One way in which these issues may be overcome is to develop membranes which have higher specific surface areas, however this can often result in compromises in other membrane properties such as mechanical strength, hydraulic permeability and pore size distribution.

Technological Developments

In light of some of the limitations of membrane chromatography detailed previously, recent literature has outline some areas in which developments are required in order to make membrane chromatography a viable alternative bioseparation technique to conventional packed bed chromatography.[89]

Improved Process and Equipment Design - As stated previously, inlet flow distribution is an area in which considerable developmental effort must be concentrated. Inefficient flow distribution in membrane chromatography systems can lead to adsorbent under-utilisation and non-optimal separation performance. Low binding capacities are currently a major limitation of membrane adsorbers and this limitation is further exacerbated by the problems encountered regarding inlet flow distribution. As a result, it can be foreseen that improvements in the design of membrane chromatography systems, including the design of the membrane capsules themselves will be a key area of future research for this particular alternative bioseparation technique.

Development of New Membranes - Lower binding capacities as a result of relatively lower specific binding surface areas compared to conventional chromatographic media are currently a major limitation for membrane chromatography. As a result considerable research effort must go towards the development of new membranes which exhibit higher capacities and improved operational properties. It is possible that since the feasibility of using membranes as chromatographic media has been demonstrated, the majority of this research effort will go towards the development of membranes designed for highly specific purposes.

Potential Applications

- To date, membrane chromatography has been shown to be capable of affecting the successful bioseparation of a range of different biomolecules ranging from monoclonal antibodies to plasmid DNA. Membrane chromatography has also been shown to be compatible with a number of separation chemistries exhibited by conventional packed bed chromatography resins including ion exchange, hydrophobic interaction, reverse

phase and affinity separations. Of these, affinity based separations using membrane adsorbers seems to be the most widely reported.[89]

Affinity based separations include the use of a number of different ligands, which can be broadly classified into four groups:

- Immunoaffinity ligands
- Protein A or G
- Low-molecular-mass ligands
- Other ligands

It should be noted that the term "affinity membranes" is applied by many researcher to all membranes which exhibit any type of adsorptive behaviour, regardless of the actual separation chemistry used. Due to the relative sizes of the pores present in chromatographic membranes compared to those found in conventional chromatography media, they are particularly suited for the capture of large protein molecules, typically with molecular weights greater than 250,000kD. Such proteins are often unable to penetrate the pores of particulate media and as a result, for such components, membrane adsorbers will often display higher relative binding capacities than their conventional counterparts. The ability of membranes to capture larger product molecules draws parallels to monoliths which are also more suited to this purpose. Regarding binding capacities, as detailed previously, membrane adsorbers typically display lower binding capacities for small protein molecules than conventional packed bed resins. As a result it may be argued that membrane chromatography is more suited to processing large volumes of liquid containing low concentrations of product. High process volumes are not a problem using membrane chromatography due to the high feasible mobile phase flow rates which can be used. Also if the product is only present at a low concentration, then the limited binding capacities of the membranes may also not be of an issue. Alternatively, membrane chromatography may be used to capture small amounts of impurities from a product enriched feed stream, meaning that it may most likely be used at later stages of the process train.[92]

Summary

Membrane chromatography is an alternative form of chromatographic separation, offering potentially higher process productivities and throughputs than conventional packed bed operations. This higher throughput is due to the higher mass transfer rates, made possible by the elimination of pore diffusion processes from the transport phenomena. As a result membrane chromatography can be seen as an alternative bioseparation technique which allows for some of the potential limitations of packed bed chromatography to be overcome. There are however currently several issues associated with the technology which may go some way towards explaining why the membrane chromatography has not been readily adopted by biomanufacturers. This major current limitation is that membrane adsorbers exhibit significantly lower binding capacity to volume ratios than conventional chromatography media. This is because membranes have a lower specific surface area available for ligand binding. This problem of low binding capacity is further exacerbated by problems with obtaining uniform inlet flow distributions, small pore size distributions and constant membrane thicknesses which when combined can all lead to adsorbent under-utilisation. In this way, many parallels can be drawn between monoliths and membrane chromatography. Both however are relatively new technologies and research effort towards the design of new membranes and membrane systems may help overcome these limitations. In its current state, given the issues concerning binding capacities, it is likely that membrane chromatography can only be used for niche processing scenarios. The ability for membrane adsorbers to handle high mobile phase velocities means that it is highly suited to capturing low concentrations of product from large process volumes. Alternatively it could be used as a polishing operation, where the aim is to operate the technique in the flow through mode, in order to capture small amounts of impurities which may still be present in the process stream at this late stage in the process train. The relatively cheap and straightforward manner in which membranes can be synthesised, compared to conventional packed bed media, also means that membranes can be used in a disposable manner which may help reduce operating costs and equipment

turnaround times.

3.5 Conclusions

This chapter has served to introduce a number of techniques which are currently being considered as potential alternatives to packed bed chromatography for the purification of biomolecules. The aim was to develop a better understanding of these various techniques and the mechanisms of separation which they each utilise. As a result details have been provided regarding the key process parameters which need to be developed in order to optimise these processes as well as information on the ways in which each technique may be performed at large scale. An attempt has also been made to review the various applications of these techniques, not only in terms of the types of products which they may be used to purify, but also the point within the process train at which each techniques may most effectively be employed.

In the process of reviewing literature data on each of these alternatives, an impression has been gained of the ways in which the potential cost and productivity issues associated with packed bed chromatography may be overcome. For example whilst the precise mechanisms used by each of the bulk separation techniques which have been detailed are quite different, they all share the characteristic of potentially scaling with the process volume rather than the mass of product which must be purified. This means that such techniques do not face the same capacity constraints as packed bed chromatography, since the size of these operations will essentially scale with the volume of the upstream process, rather than the amount of product which is being expressed. For example the same size precipitation tank will be required, regardless of whether the cell culture process is expressing 1g/L of mAb or 10g/L mAb. Similarly, field based separation techniques have an advantage over packed bed operations in that they utilise potentially cheaper consumables, allowing the cost issues associated with conventional chromatography to be overcome. Adsorptive bioseparation techniques are all essentially chromatography processes. In the case of Monoliths and Membrane Chromatography, the difference is the form of the stationary phase

support. Here the materials and structure of this support allows the utilisation of significantly higher flowrates than would be feasible in conventional chromatography, allowing for greater process productivities to be achieved.

As stated in Chapter 1, the overall aim of this thesis is to perform a quantitative evaluation of these alternative bioseparation techniques. The information presented in this chapter is generally of a qualitative nature. However it does provide a background understanding, which will serve as a foundation upon which to build further investigations. The information which has been gained in reviewing these alternatives will be used to inform the development of process models, which as shall be described in a later chapter, can be used to perform a quantitative comparison of these techniques. Such an analysis will need to account for the trade-offs between the strengths and weaknesses of these alternatives. Whilst all the alternative techniques which have been reviewed, may potentially provide benefits over packed bed chromatography, an attempt has also been made to address some of the current limitations of these technologies. Doing so has revealed the often inevitable trade-offs which occur between different process characteristics. For example CSAF and TPP may provide good performance in terms of cost and productivity respectively, but both techniques have a low feasibility for operation at scale. This significantly lessens their attractiveness. In order to assess the true potential of these techniques, it will be necessary to quantitative balance the trade-off between these characteristics. Chapter 5 details the development of a process modelling tool which enables such an analysis to be performed.

Before that however, Chapter 4 describes the results of an international survey of biomanufacturers. The intention was to gather additional information which could be used to inform the quantitative assessment of these alternative bioseparation techniques. Whilst this chapter served to outline the alternatives which have been proposed, the results of the survey described in Chapter 4, reveal the alternatives which either have actually been adopted for use in large scale manufacturing, or are being actively investigated. Such information will be useful as it will provide insight as to the technical feasibililty and scalability of these techniques as well as revealing the

techniques which are currently *perceived* to be the most attractive. The results of the survey also show the levels of importance placed on different process attributes. As stated, the quantitative analysis of these bioseparation techniques will need to account for trade-offs between different process characteristics. In order to do this, information is required regarding the level of value engineers place on each characteristic.

The information presented in this chapter therefore serves as a starting point for the evaluation of these alternative technologies. Together with the information presented in Chapter 4, the data here will allow an initial quantitative assessment to be performed, which may provide indications as to where to take further evaluations of these techniques.

Chapter 4

Changing Manufacturing Paradigms in Downstream Processing

4.1 Abstract

This chapter details a study to ascertain the degree to which bioseparation techniques which may be considered as alternatives to packed bed chromatography have so far been adopted into actual large-scale biomanufacturing. Such benchmarking information will be useful to process engineers interested in investigating the potential of these technologies. The results are based on an international industrial survey distributed to individuals involved in biomanufacturing. It yields a significant level of insight as to the current trends in downstream processing with regards to the use of alternative bioseparation techniques. Furthermore, it provides information on which alternative technologies are currently the most widely adopted as well as those which are the subject of most investigation. The major reasons for why particular alternatives have been disregarded for use at large scale provides indications as to the perceived limitations of these technologies. The survey results include details on overall trends in the biopharmaceutical industry, ranging from information regarding the types of bioproducts currently in development and manufacture, the number and

size of chromatography steps typically present in downstream processing trains as well as opinions from engineers on whether the future challenges in biomanufacturing lie either in the development of multi-product facilities or of those capable of generating multi-ton amounts of product. Analysis of the ways in which engineers feel best able to deal with these challenges, whether it be, for example, through the use of disposable technologies or alternative separation techniques are discussed. The results from two separate surveys, the first conducted in 2006 with a follow up in 2009, provide insight as to how attitudes towards the adopting of alternative purification techniques has changed over this period. Comparisons over this time period shows that there has been an increase in the level of interest and subsequent adoption of alternative technologies encompassing an increasingly wide range of different techniques.

4.2 Introduction

Chapter 2 outlined some of the concerns associated with the current MAb purification platforms, and the constraints on productivity imposed by the high reliance on packed bed chromatography processes. As discussed then, one of the proposed ways of overcoming these constraints is through the use of alternative bioseparation techniques. Chapter 3 gave a very brief outline of some of these techniques and the relative benefits and drawbacks associated with their use. Given the wide range of bioseparation techniques which have been proposed, the problem for a biochemical engineer interested in adopting an alternative into their purification process is to determine which technique is the most worthwhile investigating as it is unlikely that they would have either the time nor resources to evaluate them all. The information provided in Chapter 3 may go some way towards informing such a decision, however there is some information which cannot be so easily gleaned from the available literature. Whilst such sources do provide some insight as to the ways in which many of these techniques could be applied, information on the actual utilisation of these techniques in large scale manufacturing processes was less readily available. A complete evaluation of these alternative techniques, particularly in terms of the feasibility

of their adoption into actual biomanufacturing processes, requires an appreciation of how they are likely to behave at large scale. As is often the case, whilst inferences may be made as to the precise scalability of these techniques, until they are actually used in this manner, it is difficult to determine accurately their true suitability.

In order to address this, a study was initiated in order to ascertain the degree to which these alternative bioseparation techniques had permeated into actual large scale biomanufacturing. It was hoped that such information would be useful to process engineers interested in investigating the use of these technologies but unsure as to where to begin. At the time at which this study was started, and to a certain extent this remains true, the operation of these alternative bioseparation techniques at large scale had not been widely reported. Some literature has addressed the "industrial maturity" of these alternatives although details on the ways in which these techniques are used is limited.[29] Other publications have reviewed the wide range of techniques which are currently being considered as alternatives, and provide useful insight as to the relative advantages and drawbacks offered by each.[27, 11] However there is only a limited amount of information regarding the degree to which these alternatives are currently utilised, if at all, in large scale manufacturing processes.

On the one hand the lack of published data could be taken as an indication of the unsuitability of many of these technologies for large scale manufacturing. However it is also possible that the lack of information is a result of the reluctance of organisations to share information on new techniques that could possibly confer a competitive advantage. Paradoxically, organisations might be unwilling to commit vast resources towards the investigation of a novel bioseparation technique as it exposes them to significant risk. The high costs of process development combined with the ever increasing pressure on speed to market, means that process engineers are unlikely to take the risk of investing time and resources on investigating new technologies unless there is a reasonable certainty that their efforts will be rewarded. Instead resources used to look at a new technique may be more effectively utilised developing and optimising existing conventional operations. The situation is further exacerbated by the fact that there are a wide range of techniques available as potential replacements for

chromatography so that even if a process engineer did decide to investigate alternative technologies, they would encounter a further problem in deciding which technique(s) to look at as it would not be feasible to investigate them all.

The aim of this study was therefore to address several key questions.

Firstly, which alternative bioseparation techniques are currently deployed in industry? The successful application of a technique in an industrial manufacturing process will significantly decrease the barriers to adoption in future processes. By obtaining information regarding which techniques are already being used, this would allow the current, most industrially attractive technologies to be identified. Such data would greatly aid process engineers, looking to investigate alternative bioseparation technologies. In addition to this, it was also desired that information regarding the compatibility of these alternatives with different target bioproducts and also different host systems be obtained, as this would provide further insight for engineers looking to adopt these techniques in a range of possible biomanufacturing processes.

The second key question addressed by this study was, which alternatives have been tested by industry but have been subsequently disregarded or have not yet been implemented? The first part of the study concentrated on the use and therefore, the advantages of these alternative bioseparation techniques, the second part of the study was concerned with, possibly more importantly, their inherent limitations. Again from the perspective of aiding a process engineer in deciding which alternative bioseparation technique is the most worthwhile investigating, such information would be of a high value. For example it may be that a particular technique has been found to be incompatible with a particular expression system. If an engineer were working with this expression system then such information would allow them to disregard the technique in question from future investigations.

The study also sought to gather opinions from the bioprocessing community as to what they felt was the major challenge which would be faced by the industry in the near future and how these challenges would be best addressed, be it through the use of these alternative bioseparation techniques or some other possible approach.

These questions were addressed through the use of online surveys, which were

distributed to individuals working in biomanufacturing. The pool of respondents was selected to contain individuals working for a host of different companies, ranging from small Biotech R&D companies to Large Pharmaceuticals and Biopharmaceuticals, ensuring that the results obtained would reflect the industry as a whole. Two surveys were created, which were distributed almost three years apart. The first survey (Bioseparation Alternatives Survey I) was distributed to respondents in July 2006 whilst a second follow-up survey (Bioseparation Alternatives Survey II) was distributed in March 2009. The use of the two surveys and the time period which elapsed between their distribution dates allowed not only the key questions detailed previously to be addressed, but also to see how attitudes towards the subject matter of the survey had changed over this period.

4.3 Materials and Methods

Two separate surveys were distributed to respondents. Both surveys were developed using an online web tool (Survey Monkey^{TM}), which facilitated design and distribution of the surveys as well as collection of the results. The first survey was circulated worldwide (300 people working at 120 different company sites) to individuals selected on the basis that they were involved in the downstream processing aspects of biomanufacturing. The respondent pool comprised a wide range of organisations, including contract manufacturers, large biopharmaceutical companies as well as smaller R&D based start-ups. The surveys were not sent out en masse to all the members of the same bioprocess development team at a particular organisation. Instead, the email invitation to provide a response to the survey was aimed specifically at more senior members of staff; either team leaders or head of departments. These individuals were asked to either complete the survey or to distribute the survey to people within their organisations whom they felt were best positioned to provide a response. The second survey was distributed using the same strategy albeit with an expanded respondent pool (500 people working at approximately 300 different company sites), formed by merging the respondent pool for the first survey, with a newly acquired distribution

list. Responses to both surveys were made anonymously which helps to remove bias.

4.3.1 Bioseparation Alternatives Survey I

The first bioseparations survey contained 21 questions and was broken down into four major sections:

- *Section I* was concerned with ascertaining the level of importance placed on different process characteristics. Respondents were asked to rate different process attributes in terms of their relative importance. The aim was to determine whether there is a link between the process characteristics most valued by a process engineer, and the use, or lack thereof, of alternative bioseparation technologies. The information gained from this section of the survey would also then go on to form a key part of a further study, seeking to quantitatively evaluate these bioseparation techniques (see later work in Chapter 5)

- *Section II* was concerned with determining the "alternative" bioseparation techniques currently used by respondents in their biomanufacturing processes together with the associated performance related characteristics, such as yield and purification factor. Respondents were asked to identify any "alternatives" currently used in their manufacturing processes. Data was then collected to describe the performance of these alternative techniques in terms of process yield, purification factors, feed type to which the techniques were exposed and the position of these techniques within the process train. The aim of this section was to determine the most popular alternatives currently being used, and also to ascertain the typical level of performance expected of them

- *Section III* was concerned with identifying alternatives not currently used in biomanufacturing, but which had been evaluated in the past. Respondents were asked to identify techniques, which had either been disregarded or had not yet been implemented. Respondents were then asked to define the performance of these processes, in terms of a common set of characteristics as identified

in Section II of the questionnaire. The aim was to determine the factors and process characteristics limiting the use of certain process options in large scale manufacturing processes. By comparing the responses received for Section II and III of the survey it was hoped that conclusions could be drawn as to the most suitable operation of these bioseparation techniques. For example if a particular technique has been successfully employed as a primary capture step but had been disregarded when evaluated as a polishing operation, then it might be sensible to assume that the resolution of the process is such that it is most suitable operated earlier on in the process train.

- *Section IV* was concerned with gathering general opinions on alternative bioseparation techniques. Respondents were asked to give general feedback on not only the survey itself but also the subject matter the survey was regarding. The aim was to obtain an overall picture of how process engineers felt about the need for a downstream processing paradigm shift, how close such a shift is to realisation, and what they think are the major obstacles to this shift occurring.

4.3.2 Bioseparation Alternatives Survey II

Essentially a streamlined version of Bioseparation Alternatives Survey I, this second survey was formed by removing sections and questions from the previous questionnaire which were either no longer needed (Section I), or had a low response rate (questions regarding process yields and purification factors from Sections II and III). As a result the second bioseparations survey was significantly shorter than the first containing only 10 questions, and was split into 2 major sections.

- *Section I* asked respondents for details on their manufacturing process, including details such as the product being manufactured and the host system used to generate this product. Respondents were also asked to identify any "alternative" bioseparation techniques currently employed in their manufacturing process, or which are either being or have been investigated.

- *Section II* asked respondents for their opinions on what they thought would be the future challenges for downstream processing and the best ways in which to address them. This section was included in order to gain a perspective on the most current opinions on alternative bioseparation techniques, and their relative attractiveness compared to other possible approaches to overcoming the perceived limitations of the current purification platforms. By comparing the results of this section to those obtained from Section IV of the first survey, it was hoped that an impression could be gain of how opinions had changed over the time, which had elapsed between the distribution of the two surveys.

4.4 Results and Discussion

The results of both surveys are presented together in order to gain a perspective on possible trends in the industry.

4.4.1 Confidence Levels and Intervals

Surveys may generally be characterised by a number of key parameters, which help to describe the accuracy of its results. Since surveys are used to gain information on a population, based on taking a sample of information from a smaller group from within that population, any results obtained will all have a certain level of uncertainty associated with them. For this reason, survey results will often be quoted with a corresponding confidence interval and confidence level.[93, 94]

The confidence interval is a plus/ minus value, which is used to establish the range within which the true result would fall, were the entire population rather than a small sample, to be surveyed. For example a survey of a sample group shows that 30% of respondents gave a particular answer. This result has a confidence interval of ±5%. This implies that if the entire population were to be surveyed, the percentage of the population that would give the same response would be between 25% and 35%. The confidence level then defines the certainty with which this statement is made. For example a confidence level of 95% would imply that based on the results of the survey,

it is 95% certain that were the entire population to be surveyed, the percentage of respondents giving this particular response would be between 25% and 35%.

The confidence interval and confidence level are both determined predominantly by the size of the sample group, which is surveyed, relative to the size of the population they are being taken to represent. Larger sample sizes will invariably decrease the confidence interval and increase the confidence level. For example if 100 individuals are being surveyed in order to determine the opinions of a population of 1,000,000, the confidence interval will generally be higher and the confidence level lower, than if the sample group were made up of 10,000 individuals.

The population variance or percentage of respondents that pick a particular response will also have an impact upon the confidence interval and confidence level. For example, if 99% of respondents from the sample group choose option A and only 1% choose option B, the confidence level of 99% of the whole population choosing option A will be relatively high, regardless of the relative sample and population sizes. However if 51% are found to choose option A and 49% choose option B, the associated confidence levels will be much lower.

Equation 4.1 describes the relationship between the sample size (n) the population size (N) and the corresponding confidence levels.

$$n \geq \frac{Np(1-p)}{(N-1)D + p(1-p)} \tag{4.1}$$

Here the parameter p describes the population variance, and reflects the popularity of a particular response amongst respondents. As described previously, confidence levels are higher if a large proportion of respondents provide the same response. In the absence of additional information, p may simply be taken as the maximum allowable variance of 50%. Otherwise the percentage of respondents that give a particular response may be used in order to determine the associated confidence interval and confidence level.

The parameter D is the confidence parameter and is defined by the confidence interval (CI) and the confidence level as described by Equation 4.2

$$D = \frac{(CI)^2}{Z^2} \tag{4.2}$$

This confidence parameter is essentially a ratio between the confidence Interval and the confidence level, represented by the parameter z, which is the number of standard deviations, relative to the mean of the standard normal curve, corresponding to the confidence level. In a data set, which has a normal distribution, the number of values falling within certain multiples of standard deviations (s) can be described. For example in a standard normal distribution, approximately 68% of values will fall within 1 standard deviation or 1 sigma (1σ) of the mean, 90% will fall within 1.645 sigma (1.645σ) and 95% will fall within 1.96 sigma (1.96σ). This continues and by the time 6σ is reached, 99.9999998% of the values will have been accounted for. This therefore means that it is 68% certain that a value from the data set will fall within 1 standard deviation of the mean, and that it is almost 100% certain that all values will fall within 6 standard deviations.

The confidence level can therefore be used to determine the z value to be used in Equation 4.2. Generally speaking surveys will typically be performed based on attaining a confidence level of at least 90%, in which case, z would take a value of 1.645, or even a confidence level of 95%, in which case, z would take a value of 1.960. Confidence levels greater than 95% may be aimed for, but attaining such levels of certainty, unless the total population is very small, will require very high sample sizes.

A total of 77 responses were received for the first survey and 111 were received for the second survey. Based on these sample sizes and using Equations 4.1 and 4.2, it should be possible to determine accurately the confidence intervals of the results presented based on a particular confidence level. However such a determination requires knowledge of the population these samples are being taken to represent.

The primary aim of these surveys was to ascertain the level to which alternative bioseparation techniques have been adopted into current bioprocesses. As a result, the population may be considered to be the number of bioprocesses, which are being used

for commercial manufacture of a bioproduct, as well as those which are in sufficiently advanced stages of process development.

As stated, care was taken to target the invitations to complete the survey, towards only a few individuals at different organisations. Since the survey contains questions regarding specific details of the respondents manufacturing process, it was hoped that distributing the survey in this manner would serve to prevent duplicate responses being received. For example 10 respondents may have identified the use of Expanded Bed Adsorption Chromatography. However if all of these 10 respondents worked together on the same process, then the frequency of use of EBA would be artificially inflated, thereby skewing the ensuing analysis. Respondents were also asked to complete the survey with regards to a single process. Those involved with multiple processes were asked to complete separate surveys for each process. Based on this rationale, each survey response may be taken to represent a single sample from the desired population.

Figure 4.1 shows the impact population size has upon the confidence intervals of data, which may be obtained from the two surveys. These confidence intervals are based upon the sample sizes of 77 for the first survey and 111 for the second, with a population variance, p-value of 50%. These confidence intervals are therefore based on assuming the maximum allowable variance, representing therefore a worst-case scenario.

The chart in Figure 4.1 shows that the confidence intervals increase as the confidence level and population size are increased. It can also be seen that the confidence intervals associated with the first survey are larger than those of the second due to the smaller sample size. As stated previously the target population is the number of bioprocesses, which are being used for commercial manufacture of a bioproduct, or are in advanced stages of process development. Whilst the precise size of this population is difficult determine, it is possible to generate a rough estimate based on available data.

In 2008, a report from The Pharmaceutical Research and Manufacturers of America (PhRMA), reported that approximately 600 bioproducts were currently in Phase

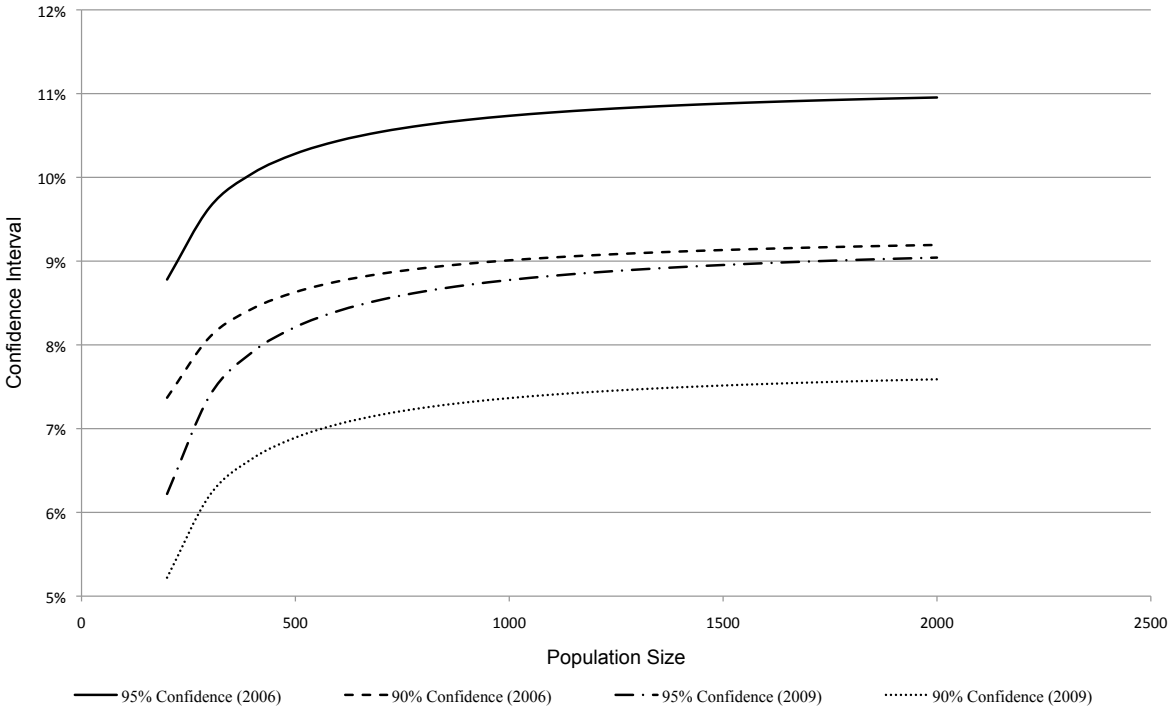


Figure 4.1: Chart showing the impact of population size upon the confidence intervals associated with data from the 2006 and 2009 surveys, based upon confidence levels of 95% and 90%

I, II or III development.[95] This group of bioproducts comprised a range of different biomolecules designed to treat a wide range of indications. By the same time point, a report from Bioworld estimated that 275 biotech drugs had gained regulatory approval.[96] Collectively then, the target population size would then seem to be approximately 875. Using this population size, the confidence intervals for the results obtained from the first and second survey may be calculated to be 9% and 7% respectively.

4.4.2 Importance Weighting

In the first section of the first survey, respondents were asked to rate different process attributes in terms of their relative importance. The attributes were broken down into 5 categories: performance, ease of operation, process economy, ease of process development and ease of scale-up. Each category was described by attributes relevant to that particular process characteristic. The different process attribute groups and

the attributes contained within each one are presented in Table 4.1.

Table 4.1: Ranking of process attributes and process attribute groups based upon the level of importance associated with each

Attribute Importance Rankings					
	1st	2nd	3rd	4th	5th
	Performance*	**Ease of Scale-Up***	**Ease of Operation***	**Process Economy***	**Ease of Development***
1st	Yield	Large Scale Operation	Ease of Control	Raw Materials Cost	Scale Down Models
2nd	Purification Factor	Scale-Up Rules	Handling Requirements	Consumables Cost	Performance Parameters
3rd	Capacity	Scale Dependent Parameters	Labour Requirements	Labour Costs	Ease of Validation
4th	Throughput	Large Scale Equipment	-	Utilities Cost	Generic Process Parameters
5th	-	-	-	Equipment Costs	Low QC Effort
6th	-	-	-	FCI	-

*Indicates attribute group.

Ratings were scaled between 1 and 5, with a rating of 1 representing a low level of importance and a rating of 5 representing a high level of importance. Ratings were based on the relative impact each attribute and attribute group would have on the decision as to whether or not a bioseparation technique would be considered for use in a manufacturing process. The relative importance ratings given were expected to reflect the respondents particular experiences and constraints. The aim was to therefore gather a range of opinions, to identify if there were indeed common trends across the industry, with regards to which attributes were most valued.

Results shown in Table 4.1 summarise both the importance ranking of the overall attributes groups as well as the ranking of the process attributes comprising each of the groups or categories. The ranking of the attributes and attribute groups was based upon the average importance rating (data not shown) given by the respondents. On average, respondents felt that *performance* (and all the associated process attributes) was the most important process characteristic in determining whether a particular technique is adopted for use in manufacturing whilst *ease of process development* was considered to have the lowest level of influence upon the implementation of a given technique. Over 60% of respondents gave the process *performance* attribute category a rating of 5 and over 90% gave it a rating of more than 4. The high level of importance placed on process performance is understandable. If a process is not

capable of providing the level of purification or yield which is required, then it cannot be used.

The *ease of process scale up* was considered to be the second most important factor when determining whether or not to adopt a particular bioseparation technique. Almost 70% of respondents gave this attribute category a rating of 4 or 5. The result is connected with the fact that if a process is difficult to scale-up or is not suitable for use at scale, then it will automatically not be suitable for use in a biomanufacturing process.

The *ease of operation* was considered the third most important attribute group, which comprises attributes such as the ease of process control. This most likely reflects the increasing importance being placed upon implementing Process Analytical Technology (PAT) and Quality By Design (QBD) into biomanufacturing processes.

Interestingly, given the current pressures on reducing process costs, *process economy* was considered by survey respondents to be the second least important factor, with the attribute group receiving an average importance rating of approximately 3.7 This can be rationalised, since if a process is highly cost-effective and hence cheap to run but does not provide sufficient purification or yield, then it will not represent a viable process alternative. Similarly if the process is cheap but cannot be operated at large scale, then again, the cost effectiveness of the process becomes academic.

The *ease of process development* was considered to have the lowest impact upon the decision as to whether to adopt a certain bioseparation technique, obtaining a moderate average importance rating of 3. This relatively low importance ratings compared to that given for performance may be due to the fact that if a particular technique is providing the level of performance which is required, then additional resources can simply be implemented to help resolve any process development difficulties.

The ranking of the attribute groups shown in Table 4.1 is based purely upon the average importance ratings given by the survey respondents. A more robust statistical analysis using the Wilcoxon Rank Sum test[97, 98], in which the pairwise difference in importance ratings obtained for the attribute groups were compared showed that whilst *Process Performance* was indeed considered more important that

the other attribute groups (the p value was less than or equal to 0.001 when ratings for performance were compared against those for each of the other attribute groups), the differences between the other attribute groups was slightly more subtle. The *Ease of Scale-up* and *Ease of Operation* are definitely considered more important than *Process Economy* and *Ease of Process Development*. However the differences between the ratings given for the *Ease of Scale-up* and *Ease of Operation* attribute mean that a definitive distinction between the perceived importance of these two attribute groups cannot be made.

The following is a breakdown of the ranking of the attributes within each of the attribute groups (Table 4.1):

Process Performance

On average, respondents felt that *yield* and *purification factor* were the most important process attributes in terms of performance. This result is understandable since poor product yields or low purification factors will often be adequate grounds to dismiss a given technique regardless of its productivity. Given the high production costs and hence value of most biopharmaceutical products, product losses are not desirable, regardless of whether or not productivity can be maintained through raising process throughputs. It is most likely for this reason that yield is perceived as such a highly valued process attribute. The same can be said for purification factor. With biopharmaceutical products, destined for human therapy, the level of product purity is of paramount importance and any separation technique incapable of meeting such requirements, regardless of the other process attributes, cannot be used in a manufacturing process.

Ease of Scale-Up

Over 80% of respondents agreed that if a technique has previously been successfully operated at large scale, then the probability of further successful scale-up is increased significantly, giving this attribute an importance rating of 4 or 5. The availability of *established scale-up rules* and also a low number of *scale dependent critical process*

parameters were also seen as important in determining the ease of process scale-up with over 90% of respondents giving these attributes a moderate to high importance rating. Conventional unit operations are generally scaled via a set of rules which provide a guideline as to how the process should be scaled-up. If such rules already exist and have been successfully used in the past, this will significantly increase the ease of process scale-up. Whilst scale-up is normally done by maintaining a set of process conditions, it may not always be possible to maintain all of the conditions involved in a particular operation. As a result the complexity of process scale-up may be greatly reduced if the operation only involves a small number of scale dependent process parameters, explaining the relative levels of importance placed on these attributes. The *availability of large scale equipment* attribute was regarded to be of the lowest relative importance. This is likely to be because the availability of large scale equipment will only affect the scale-up of a particular process to a certain extent. Lack of suitable equipment represents a logistical problem, which may be resolved simply by increasing expenditure to buy custom-made equipment.

Ease of Operation

In terms of determining the ease of process operation, respondents felt that *ease of process control* was the most important process attribute, a sentiment which was shared by a large fraction of the biomanufacturing population, giving this particular process attribute an average importance rating of approximately 4. With regards to the other two attributes which make up this category, the importance ratings given for the *low levels of handling* and *low labour requirements* attributes were very similar with both receiving an average importance rating of approximately 3 (low handling requirements had a slightly higher average importance rating). This indicates that the ease of process control is of much greater concern to biomanufacturers than the amount of labour and handling required to run the process. This is understandable since greater levels of process control also facilitate process validation and conversely if a technique cannot be sufficiently controlled so as to give a product stream of consistent quality, then it cannot be used for manufacture regardless of how little

labour is required to operate it.

Process Cost and Economics

The average importance ratings for all of the attributes in this category were highly comparable, indicating that, in general, respondents did not think any individual attribute was more important than any other with all attributes receiving an importance rating ranging from 2.5 to 3 with the ratings given for low raw materials costs and low consumables costs at the higher end of this range. Surprisingly the *cost of equipment* required for a particular process has an average rating (2.6) at the bottom end of the range, a trend reflected by the importance rating given by respondent to a technique having a low associated level of *fixed capital investment* (2.7). These results indicate that biomanufacturers will tend to be more concerned with how much it will cost to operate a process than how much it costs actually to set the process up in the first place. This may be due to the fact that investing capital for equipment purchase, plant construction and installation is unavoidable. The fixed capital investment will be primarily determined by the scale of the operation, itself a function of the level of demand for the product. These are factors, which are outside the process engineers control. Whilst these distributions indicate that biomanufacturers place greater importance upon operating cost components than the fixed capital investment, the actual order of prioritisation of these two factors is likely to depend upon the kind of organisation being considered. Small start-up companies may have low levels of capital available and would regard the initial investment required to construct their manufacturing facilities as being of paramount importance. Alternatively a contract manufacturer may not place a great level of importance on the fixed capital investment required as the acquisition of additional equipment will be repaid by the income obtained from customers accessing the equipment.

Ease of Process Development

Almost 80% of respondents thought that having a sufficient *understanding of performance driving parameters* and having *applicable scale down models* available were the

most important prerequisites for an increase in the ease of process development, giving these two attributes a rating of 4 or 5. A comprehensive knowledge of the underlying mechanisms of separation will allow the process engineer to determine which process parameters need to be altered and tested in order to optimise the process. Similarly since most process development work should preferably occur at a small scale this necessitates the need for small scale experimental equipment which can reliably predict the performance of a particular process at large scale.

Over 60% of respondents gave the *ease of process validation* attribute an importance rating of 4 or 5. This is again understandable as validation, a costly and time consuming activity, is something which must be constantly considered when performing process development. The *availability of generic process conditions* for a particular technique was considered by the majority of respondents to only be of a moderate importance. This is perhaps indicative of the current approaches used for process development. In particular, the emergence of high throughput screening techniques combined with the proliferation of design of experiment (DOE) developmental approaches means that protracted trial and error style iterative process development can be avoided. The average importance rating given for the *Low QC Effort* attribute (3) likely relates to the fact that quality control is an inevitable feature of process development.

The ranking of the attributes listed in Table 4.1 can be used in a Multi-attribute decision making (MADM) based analysis of these various separation techniques. A detailed description of this approach is presented in Chapter 5.

4.4.3 Organisation Types

Respondents were asked in the second survey, but not the first, the type of organisation for whom they worked for. Such information was felt to be useful as it allows differences in attitudes and opinions between different types of companies to be observed.

As stated previously responses, for both surveys were made anonymously. The anonymous nature of the survey responses was felt to have been an important aspect

as it was thought that this would encourage more people to respond. For example an engineer may be more comfortable sharing information if they knew that the information disclosed could not be tracked back to either them or the company for which they worked. In order to preserve this anonymity, whilst also obtaining the desired information, rather than being asked to explicitly name the organisation for which they worked, respondents were instead asked to identify from a broad list of organisation types, the one which they felt best described the company for which they worked.

Figure 4.2 shows a breakdown of the different types of organisations from which responses to the survey were received.

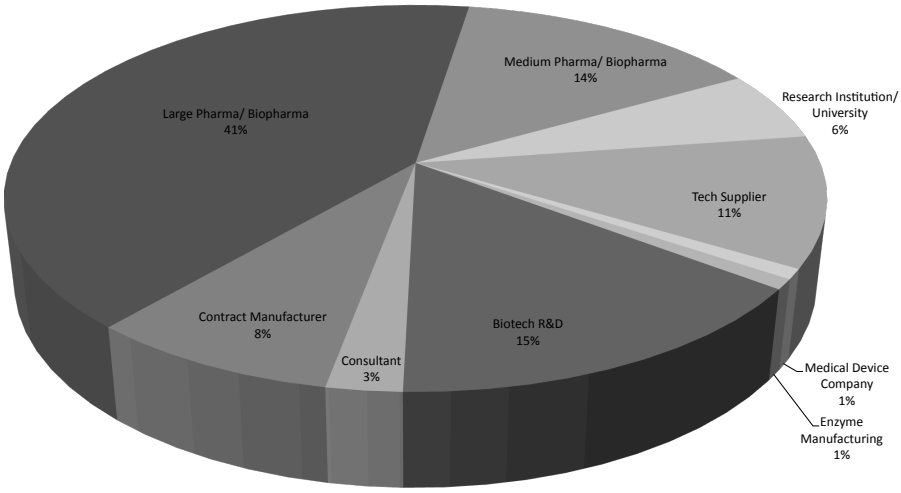


Figure 4.2: Breakdown of survey responses in terms of the type of organisations for which the respondents worked

Figure 4.2 shows that the responses to the survey were predominantly received from individuals working in large Pharmaceutical or Biopharmaceutical companies, making up a total of 41% of the responses received for the survey. Medium to small pharmaceuticals and biopharmaceuticals and Biotech R&D start-up companies make up the next largest proportion of the respondents, with 14% and 15% of the share,

respectively. Technology Suppliers and Contract manufacturers made up the third tier of organisations, in terms of the proportion of respondents that worked for these types of companies. Technology suppliers consisted of organisations which provided equipment and materials such as centrifuges and chromatography resins, for use in biomanufacturing, but themselves did not manufacturer any biotherapeutic.

Whilst the results in Figure 4.2 would imply that there is no extreme bias in the results, reflecting the opinions of individuals working for only one type of organisation, the relatively large proportion of responses received from Large Pharmaceuticals and Biopharmaceuticals, means that there must be some slight bias in the results towards them. Such a bias is however, likely to be unavoidable. The nature of the pharmaceutical and biopharmaceutical business means that the data shown in Figure 4.2 is most likely reflective of the actual segmentation of the industry. Large Pharmaceuticals and Biopharmaceuticals will simply have a greater presence in the field of bioprocessing as a whole, both in terms of the number of products which they have in the market and in pipelines, as well as the number of individuals whom they have working for them. If each survey response is taken to represent a single bioprocess, then it is likely that these larger organisations would have a greater number of processes either in operation or development, thereby explaining the larger proportion of responses received from these organisations.

One concern when dealing with surveys is that the responses are biased towards one particular subset of the population thereby skewing the results obtained. A good example of this would be a workload survey in which office workers are asked how busy they were. Such a survey, would likely only be completed by people who were not busy and had the time to complete the questionnaire and as a result, the responses might indicate that a high proportion of the office were underworked, when in reality the complete opposite may be true. The concern then is that a similar bias may be present in the results of this survey. For example, it might be that individuals working for smaller biotech companies were for one reason or another, less inclined to provide a response to the survey. One way of testing this is to compare the individuals to whom the survey was sent to those that actually provided a response, in terms of

the organisation for which they worked. The list of individuals to whom the survey was sent was basically formed from a combination of the University College London, Department of Biochemical Engineerings list of contacts and that of an international bioprocessing conference. Based on its source, this group of potential respondents and the organisations for which they work may be taken as a reflective of the industry as a whole.

Figure 4.3 shows a comparison of these potential respondents and those that actually provided a response in terms of the organisations for which they worked.

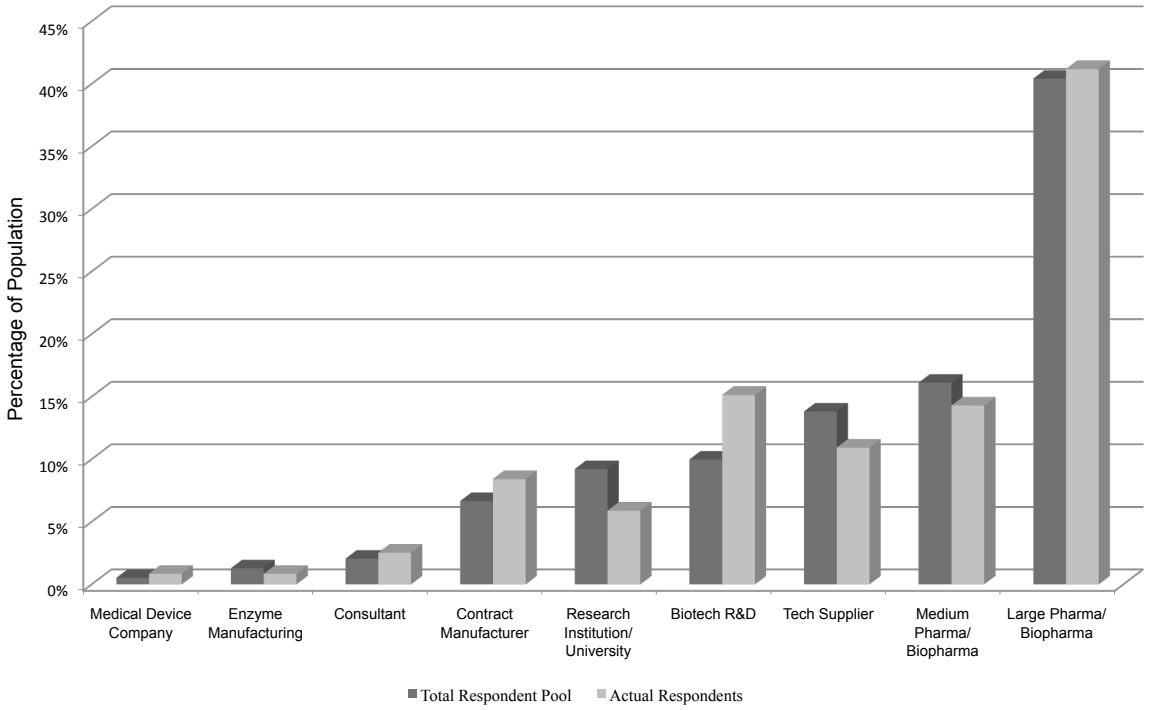


Figure 4.3: Chart showing the percentage of individuals, to whom the survey was sent, working for different types of organizations as compared to the percentage of responses actually received from individuals working for each type of organization.

The agreement between the two sets of data would imply that any bias which exists in the data obtained from the survey response is a result of the composition of the original respondent pool to whom the survey was sent, rather than there being any inherent apathy from people working for particular types of organisations. Furthermore, if it is assumed that the composition of this respondent pool is a true reflection of the actual segmentation of the industry, then the results, which have been

obtained, may be interpreted as being representative of the biotech/biomanufacturing sector as a whole.

4.4.4 Products of Manufacture

Both surveys asked respondents to identify the product, which their process manufactures, or is being developed to manufacture. Figure 4.4 shows a comparison of the breakdown of products, based on the responses to the surveys distributed in 2006 and 2009.

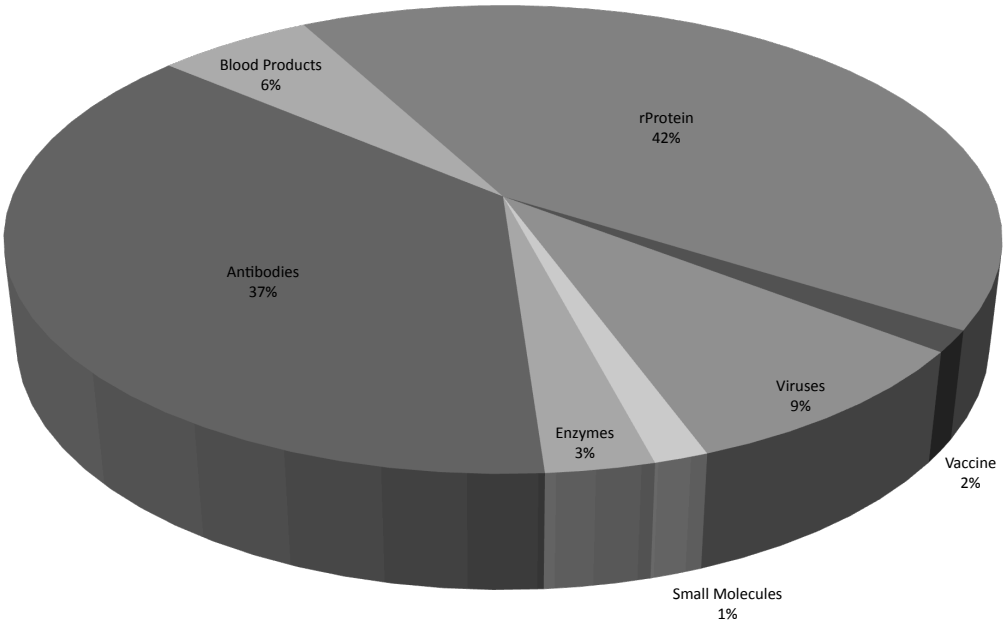
Comparing the two charts shown in Figure 4.4 it can be seen that the two major product types both in 2006 and then in 2009 were antibodies and recombinant proteins (rProteins).

In 2006, almost 90% of the respondents provided an answer to this portion of the survey whilst in 2009 the figure was over 95%. The high proportion of non-null responses indicates that the product breakdown shown in Figure 4.4 is a true reflection of the product profiles across the biotech sector.

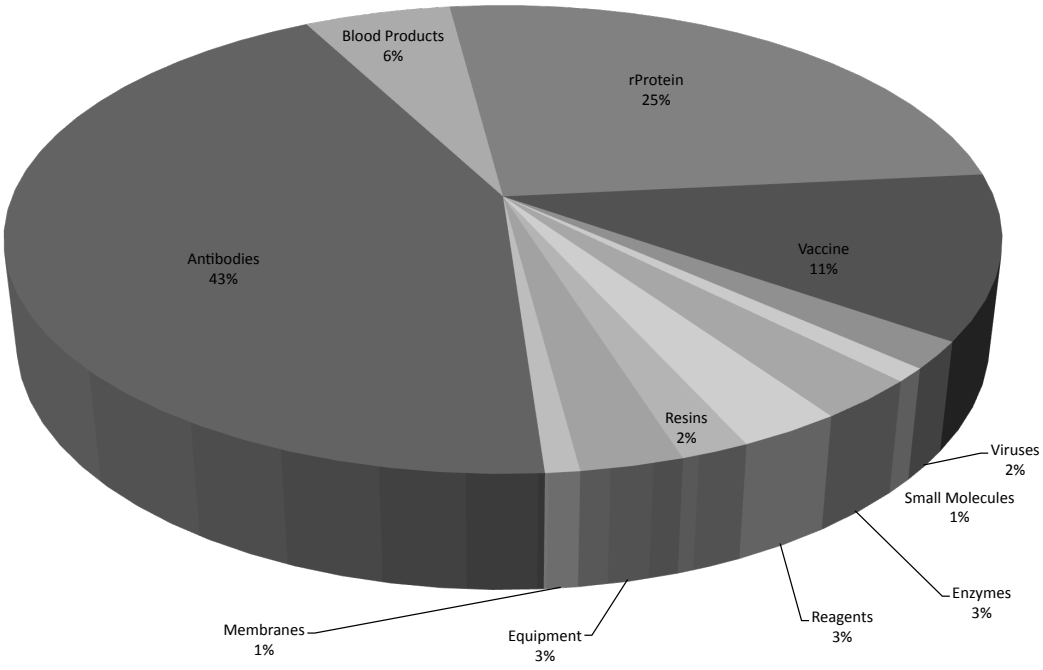
Comparing the two charts shown in Figure 4.4, it can be seen that whilst rProteins were the predominant product class in 2006, with a percentage share of 42%, in 2009 this share decreased to 25%. Antibodies and Vaccines meanwhile have experienced an increase in their percentage share over the same period, possibly reflecting the shift of focus in the biopharmaceutical industry from first generation recombinant proteins such as erythropoetins and insulins to second and third generation biotherapeutics, as represented by the antibodies and vaccine product classes respectively.

4.4.5 Alternative Technologies Used

Respondents to both surveys were asked to identify alternatives bioseparation techniques currently used in their manufacturing process, either choosing them from a predefined list (Table 5.1), or providing details of an technique which they considered to be an alternative to traditional packed bed chromatography. The list of pre-defined techniques was based on those outlined in a review publication of alternative biosep-



(a) 2006



(b) 2009

Figure 4.4: Comparison of the types of product being manufactured by survey respondents in 2006 and in 2009

aration technologies.[29]

Table 4.2: Pre-defined alternative bioseparation techniques listed in the surveys

Bulk Separations	Field-Based Separations	Adsorptive Separation
Aqueous Two Phase Extraction (*ATPE*)	Affinity Filtration (*Aff. Fil*)	Monoliths (*Mono*)
Bulk Protein Crystallisation (*Cryst*)	High Performance Tangential Flow Filtration (*HPTFF*)	Expanded Bed Adsorption Chromatography (*EBA*)
Three Phase Partitioning (*TPP*)	Controlled Shear Affinity Filtration (*CSAF*)	Membrane Chromatography (*MC*)
Macroligand Facilitated Three Phase Partitioning (*MLFTPP*)		
Primary Effect Affinity Precipitation (*PEAP*)		
Secondary Effect Affinity Precipitation(*SEAP*)		
Magnetic Adsorbent Particles (*MAP*)		

Abbreviations for each technique, as used in the main text of the paper, are shown in *italics*

In 2006, 42% of the survey respondents indicated that they utilised at least one alternative bioseparation technique in their manufacturing process. In 2009, this figure has increased to 60%. Accounting for the confidence intervals associated with these figures (9% and 7% for the first and second survey respectively) this result indicates that there has been an increase in the uptake and adoption of these technologies over the three-year interval between surveys (data not shown).

Figure 4.5 shows the number of alternative techniques utilised by the survey respondents for both the 2006 and 2009 surveys. Error bars indicate the confidence intervals associated with the data. From this it can be seen that of all the respondents that identified the use of alternatives in their process, the majority (50 – 60%) only utilise one such technique in the purification train.

This trend possibly reflects the resource intensive nature of adopting an alternative technique, as with the constraints associated with process development, manufacturers simply do not have sufficient time to evaluate and develop more alternative unit operations.

Figure 4.6 compares the alternative bioseparation techniques used by the survey respondents in 2006. The error bars again represent the confidence intervals associated with the data as described previously.

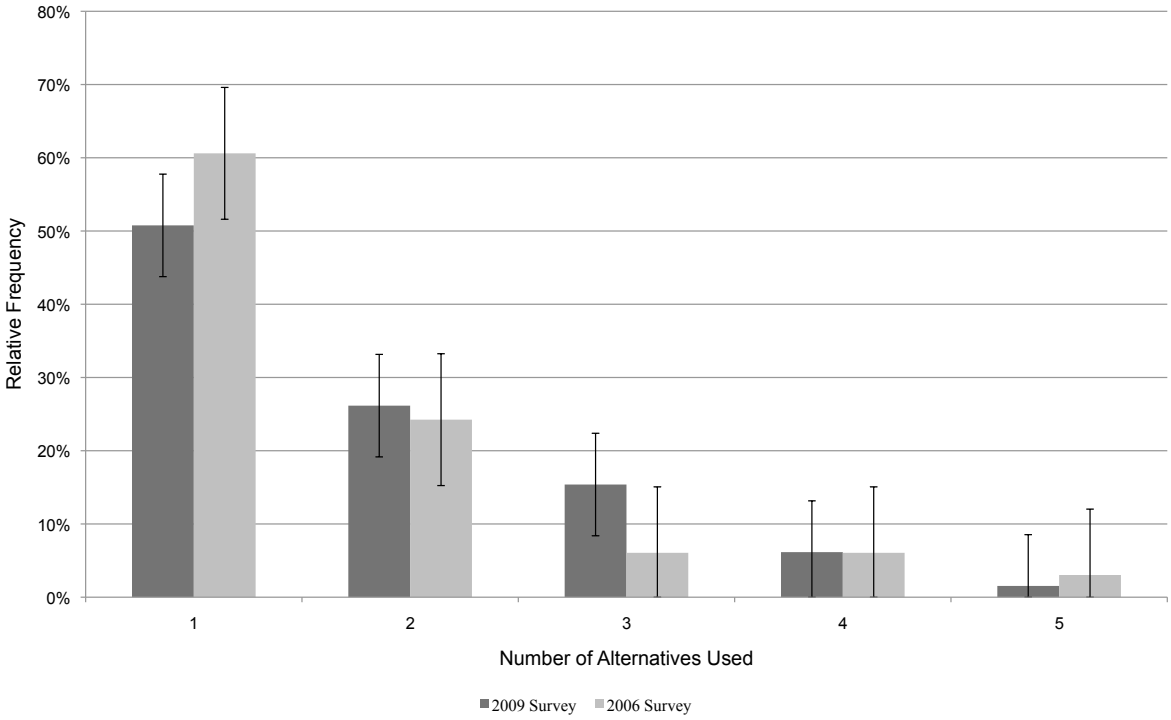


Figure 4.5: Number of alternatives utilised by individual respondents with error bars represent the associated confidence intervals

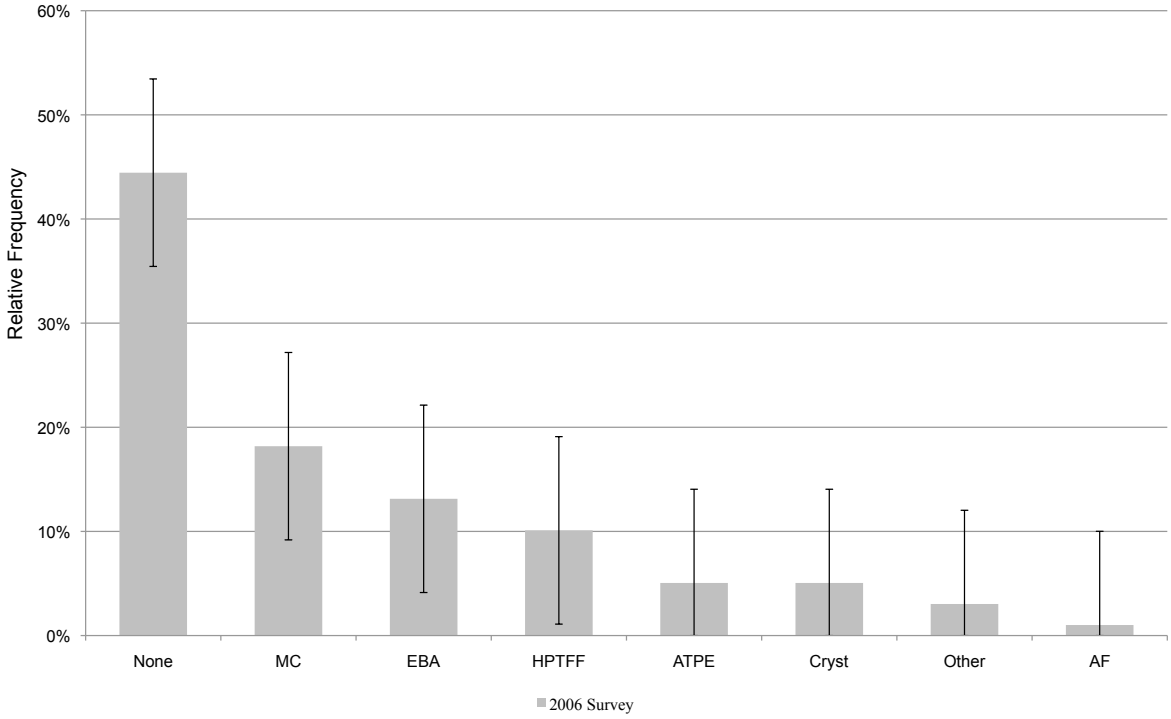


Figure 4.6: Comparison of alternative bioseparation techniques used in manufacturing in 2006 with error bars represent the associated confidence intervals

From results shown in Figure 4.6, the alternative techniques utilised in manufacturing processes in 2006 may be broadly separated into two tiers, in terms of the frequency of their use. The top tier comprises Membrane Chromatography (MC), Expanded Bed Adsorption Chromatography (EBA) and High Performance Tangential Flow Filtration (HPTFF), whilst the bottom tier comprises Aqueous Two Phase Extraction (ATPE), Affinity Filtration (AF) and Crystallisation (Cryst). The overlapping confidence intervals means that it is not possible to definitively make the distinction between the two tiers, as it could be argued that all these alternative may have the same frequency of use, if the results are extrapolated to apply to all current manufacturing processes. However based on the responses received to the survey, it does appear that MC, EBA and HPTFF are the most popular alternatives and this popularity is fairly understandable.

Membrane Chromatography and HPTFF are both separation techniques, which are based upon established downstream processing technologies. In the case of Membrane Chromatography, the principles of operation are fundamentally similar to those used for conventional packed bed chromatography, the only difference being the form of the stationary phase which is used.[89] Similarly, HPTFF is essentially a highly optimised form of conventional tangential flow filtration in which the process is operated under pressure dependent flux conditions, with the use of co-current flow on the filtrate side of the membrane to maintain optimal transmembrane pressure conditions across the entire membrane surface.[99, 100] Neither Membrane Chromatography nor HPTFF could be considered direct analogues to their conventional counterparts. Chromatography membranes typically display lower capacities than traditional media, and issues can be encountered with ensuring uniform fluid flow across the entire membrane surface.[89] Similarly the use of co-current flow in the HPTFF process provides an additional process parameter, which much be optimised and controlled whilst the potential for the use of charged membranes adds a further layer of developmental and operational complexity.[65, 64] However the underlying mechanisms of separation for these technologies are based on those of unit operations, which are commonly employed in the majority of modern bioseparation processes. The performance of

these alternative techniques is therefore predictable to a certain extent and as a result the perceived risk of utilising such techniques is reduced compared to processes such as Aqueous Two Phase Extraction (ATPE), Crystallisation and Three Phase Partitioning (TPP), which have no such grounding and as such have not experienced as frequent adoption.[33, 49, 101]

Another factor worth considering is the point of application of these alternative separation techniques. Membrane chromatography and HPTFF are both unit operations, which are designed for use at later stages of the purification train, where the purification burden is relatively low, particularly when dealing with products such as monoclonal antibodies. It could be argued based on this then that people are more willing to include these operations at a later stages in the process, wherein the lower purification demands means that the inherent risks associated with the adoption of these techniques is outweighed by the benefits. For example in a conventional MAb purification process, the final chromatography step will typically be an anion exchanger, operating in flowthrough mode. Anion exchange Membrane Chromatography presents a suitable alternative here despite the low associated binding capacities, as the aim of this step is to bind impurities which at this stage in the process should only be present in trace amounts. Furthermore Membrane Chromatography capsules may be operated at significantly higher flowrates than conventional packed beds allowing for higher throughputs. Similar arguments can be used when discussing HPTFF. Following the final AIEX chromatography step, most MAb purification processes will include a tangential flow ultrafiltration/ diafiltration step at the end of the train in order to achieve product concentration and buffer exchange into the final formulation conditions. HPTFF allows this TFF step to be combined with the final AIEX step, helping to reduce processing costs and time, whilst increasing process yields.

Of all the alternative techniques available, EBA is one of the most mature, having been investigated and developed since the early 1990s.[75] This relative maturity and the corresponding level of understanding around the technique means that as with membrane chromatography and HPTFF, the performance of EBA may be predicted to a certain extent, thereby lowering the technical risk associated barriers to its adoption.

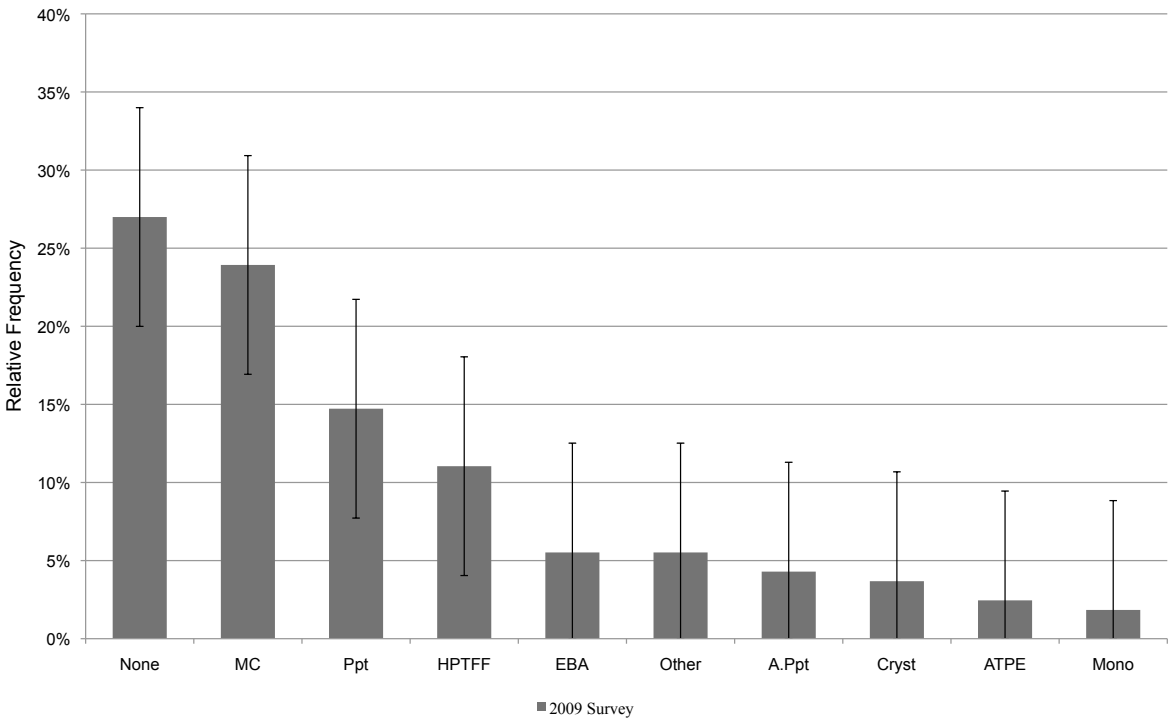


Figure 4.7: Comparison of alternative bioseparation techniques used in manufacturing in 2009 with error bars represent the associated confidence intervals

Jumping forward to 2009, Figure 4.7 shows that again, as was the case in 2006, the alternative techniques being used may be broadly separated into two tiers in terms of the frequency of their adoption. The techniques comprising each tier are also generally the same, the major difference being that EBA has moved into the bottom tier, and has been replaced by Precipitation (Ppt).

Comparing Figure 4.6 and 4.7, it would seem that the range of alternative techniques being utilised has expanded to include techniques such as Monolith based chromatography (Mono) and precipitation. Indeed almost a quarter of survey respondents identified the use of either non-specific or affinity precipitation processes in their manufacturing process, and as mentioned non-specific precipitation is now one of the most widely adopted alternative techniques. This is a fairly dramatic increase considering that in 2006, no respondents indicated the use of any precipitation processes.

The sudden and dramatic rise in the number of uses of Precipitation is slightly unusual since it would imply that almost 25 new manufacturing processes have emerged

over the past three years since the first survey, all of which utilise precipitation at some point in the purification train. It is possible that the higher incidence is due to the increased size of the respondent pool, and that the use of precipitation did not emerge in the results of the first survey as these additional individuals were not polled. This explanation however would require the vast majority of the additional respondents to all utilise precipitation in their manufacturing process. It would also imply that there was a bias in the distribution of the first survey, with it only being sent to organisations that did not use this particular bioseparation technique. An alternative explanation would be that survey respondents, rather than identifying alternative techniques which are currently being used in their manufacturing process, have instead chosen techniques which they are currently investigating and are in the process of being implemented. The latter is the more feasible explanation, however with the results available it is impossible to determine whether this explanation is true and instead the results must be taken at face value.

Regardless, the level of interest in precipitation has at least increased over the intervening years since the first survey. This rise in popularity is most likely attributable to a combination of factors. Firstly the increase in mAb titres from mammalian cell cultures, has made semi-selective precipitation of the product more feasible, allowing the product mAb to be brought out of solution using lower precipitant concentrations. This would not have been possible a decade ago with mAb titres typically in the range of tenths of a gram per litre. However with modern cell culture processes commonly expressing mAb at concentrations of 5 g/L and some even up to 10g/L, precipitation has become a much more viable process option.

The percentage share of ATPE and Crystallisation has dropped slightly between 2006 and 2009. As with EBA, this is mainly a result of an increase in frequency of precipitation use. In 2006, 5 respondents indicated the use of ATPE and 5 indicated the use of crystallisation in their manufacturing process. In 2009, these numbers changed to 4 and 6 respectively. The reason that these techniques have not experienced an increased level of adoption, may only be speculated upon, however based upon the authors own personal experience, crystallisation and ATPE are both techniques which

have issues associated with process robustness and performance. Given the high level of importance placed upon process performance, this may explain the limited uptake of these techniques.

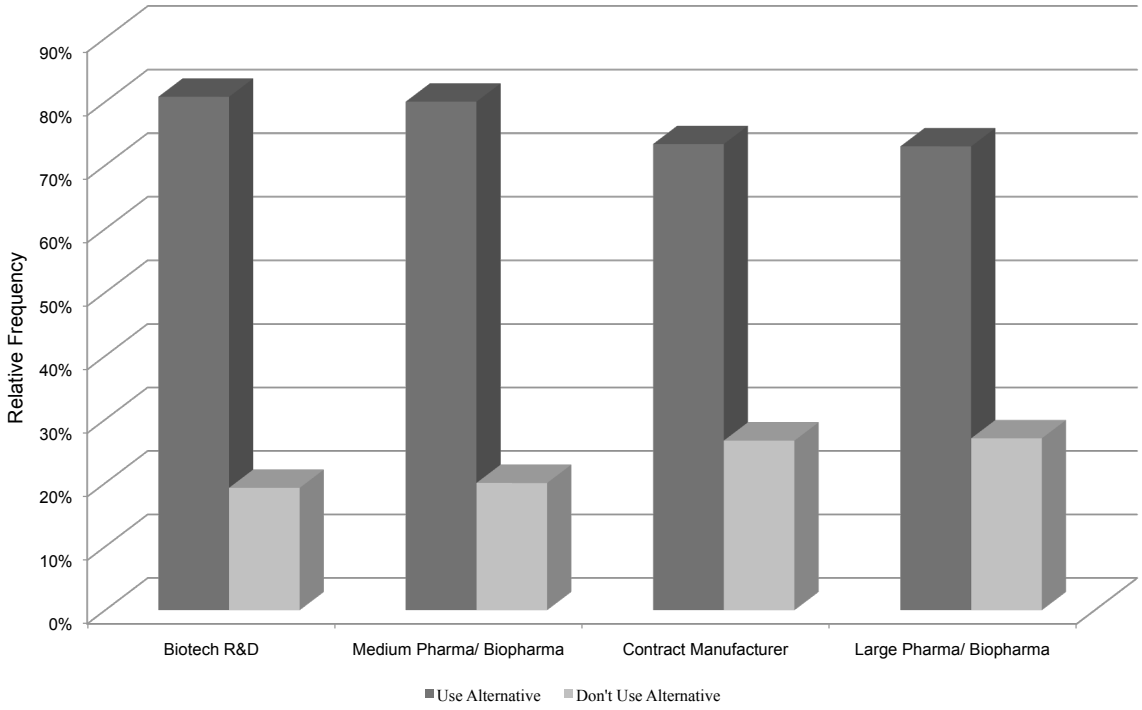


Figure 4.8: Use of alternatives by different types of organizations

Figure 4.8 shows the proportion of respondents to the 2009 survey, that cited the use of an alternative bioseparation technique in their manufacturing process, compared to those that indicated that they did not use any, broken down by the type of organisation for which the respondents worked for. This chart shows an interesting trend, as it seems that there is a very slight inverse relationship between the size of the organisation and the likelihood that they would adopt an alternative bioseparation technique. As a result whilst 80% of respondents from small R&D based Biotech start-ups cited the use of alternatives, only 70% of individuals that worked for large pharmaceutical companies indicated that they had adopted an alternative in their manufacturing process.

This trend is slightly surprising as it contradicts what might originally be expected. Larger companies would be more likely to have the necessary resources to develop and apply alternative technologies, whilst smaller companies, given its resource intensive

nature, would presumably be more inclined to take a more conservative approach to process development. The data here suggests that the likelihood of a company adopting an alternative technique is at least equal amongst all types of companies and if anything, it is the smaller companies which are more likely to take the risk of adopting alternative processing strategies. The reason for this may be due to the high costs involved in developing biological therapeutics means that smaller companies are forced to be more creative and take more risks in order to gain an advantage over their more financially secure counterparts.

4.4.6 State of Chromatography

The initial driving force for the investigation of these alternative technologies was the concerns regarding potential capacity limitations imposed by packed bed chromatography. Indeed the term alternative technologies itself is coined based on the fact that these separation techniques are potential alternatives to packed bed chromatography. Therefore it is interesting to note the impact that the utilisation of these techniques has upon the number of chromatographic steps present in the downstream purification train.

The chart shown in Figure 4.9, compares the number of chromatography steps used by survey respondents in 2006 to those used in 2009. The results from both time points are fairly comparable with an almost normal distribution emerging in both 2006 and 2009. Respondents on average, employ three separate chromatography steps within their downstream process whilst the range of these distributions shows that some respondents do not utilise any chromatographic separation within their downstream processes at all, whilst some may use more than four. It could be argued, based on this data, that there has been a slight shift towards smaller column numbers in 2009. For example in the 2006 survey, 10 respondents quoted the use of more than three chromatography columns in their purification train, whilst in 2009, only 2 respondents fell into this category.

The move towards the use of fewer chromatographic steps is representative of a general shift in biomanufacturing towards the development of more efficient purifi-

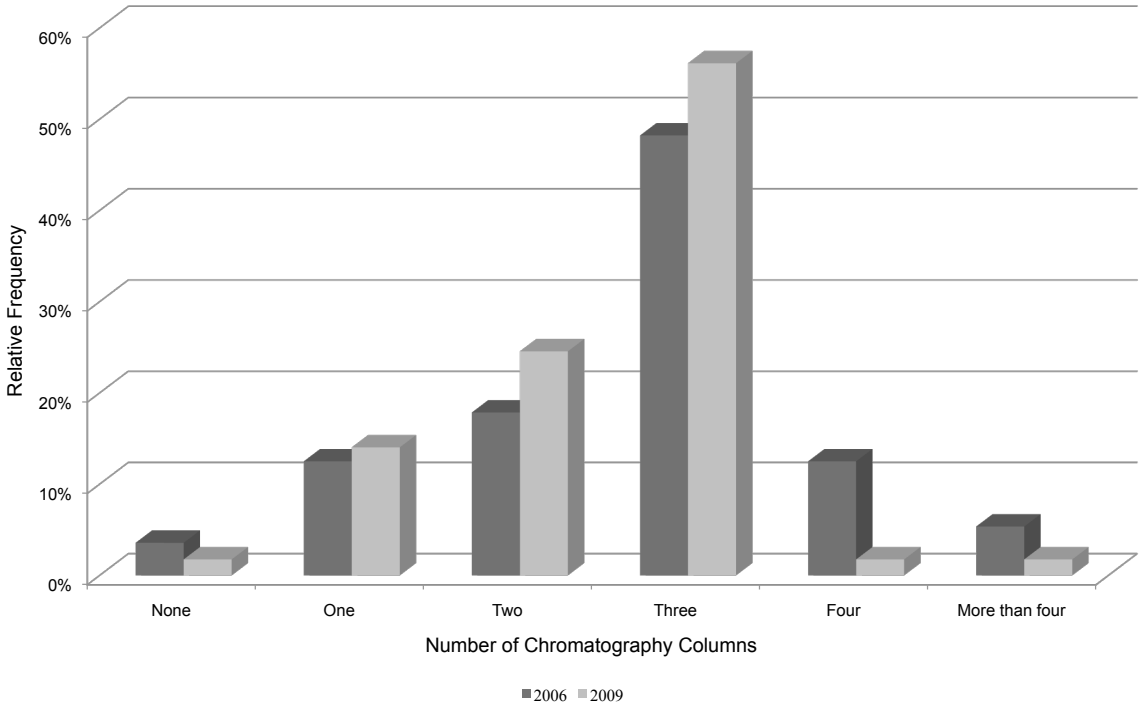


Figure 4.9: Comparison of number of chromatography steps present in downstream purification train in 2006 and in 2009

cation processes in which the maximum purification potential of each unit operation is fully realised. The trends in the development of mAb purification processes is a clear example of this, whereby the traditional three step purification platform is now beginning to give way to purification trains which contain only 2 packed bed chromatography operations.[102] This idea of increasing process efficiency is reflected in the size of the chromatography columns being used. Table 4.3 compares the volume of the first three chromatography columns used by the survey respondents in 2006 and 2009. From this it can be seen that there has been a 30% drop in the average volume of the first chromatography column, and almost a 50% drop in the average volume of the subsequent chromatography steps.

The use of smaller columns achieved through improvements in resin design (higher dynamic binding capacities) and/or platform process improvements not only reduces the consumables costs associated with the chromatography resins, but also helps to reduce the raw materials costs associated with buffers and cleaning solutions. The use of smaller columns also potentially allows for improvements in productivity to be

achieved.

Table 4.3: Comparison of chromatography column volumes used in 2006 and in 2009

	Average Column Volume (L)		
Chromatography Column	2006	2009	% Change
1	119	84	30%
2	124	64	49%
3	111	57	57%

Interestingly in both the 2006 and 2009 surveys, the use of alternatives does not have a significant impact upon the average number of chromatography steps used in the purification train. That is the average number of chromatography columns present in the process remains at approximately 3, regardless of whether alternatives are used or not. This would imply that these alternative bioseparation techniques are not being used as direct replacements for chromatography but are instead being used to supplement the purification performance of the more traditional purification train.

4.4.7 Alternative Techniques Evaluated

Both surveys asked respondents to identify alternative separation techniques which they are either evaluating, or have evaluated and subsequently disregarded. Such information is useful as it may help reveal common limitations of some of these techniques.

The results of the 2006 survey revealed that approximately 35% of the survey respondents had neither adopted the use of an alternative bioseparation technique or had evaluated one. In 2009 this figure had fallen to only 20%. These figures show the high level of interest in the use of alternative bioseparation techniques with, in 2009, approximately 80% of respondents indicating that they have at least investigated the used of one or more alternative bioseparation techniques.

Interestingly, in 2006, of the respondents that indicated the use of at least one

alternative bioseparation technique, only 15% stated that they evaluated more that one other alternative technique. The low percentage of respondents that investigated further techniques other than the ones which have been adopted into their manufacturing process, reflects the large time and resources requirements associated with adopting new technologies.

Figure 4.10 shows a breakdown of alternative techniques, which either are, or have been in the past, evaluated by the respondents to the 2006 survey. Error bars show the associated confidence intervals.

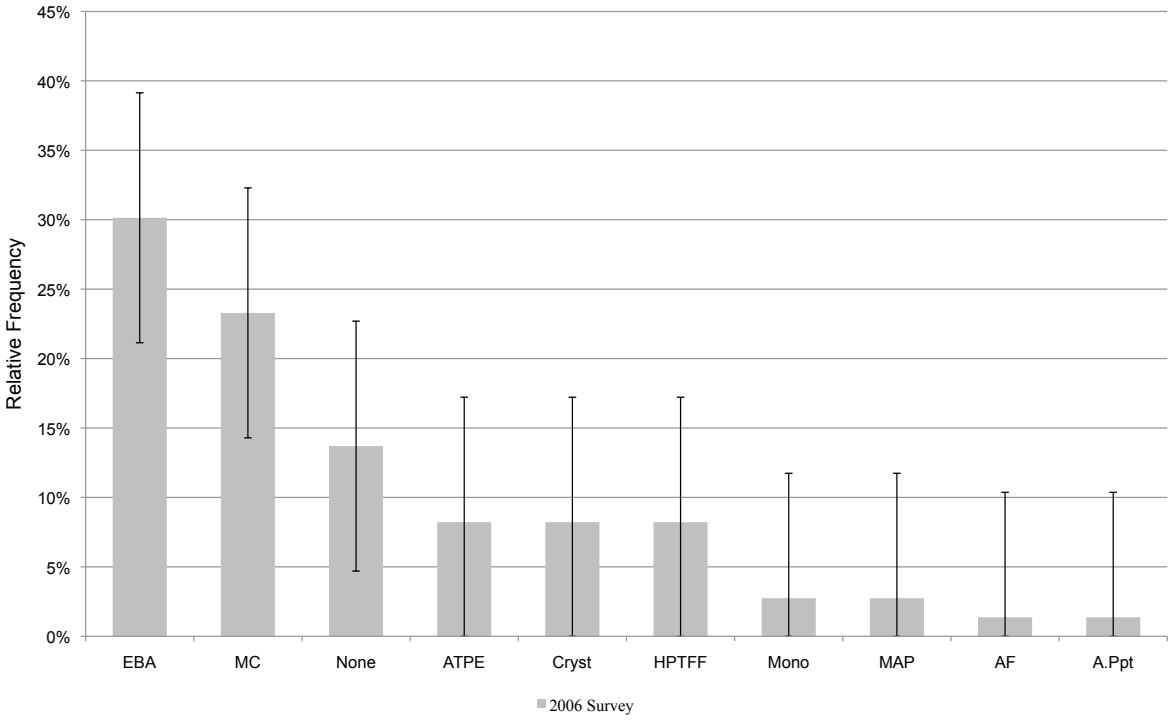


Figure 4.10: Alternative bioseparation techniques which are either being evaluated, or have been evaluated in the past by respondents to the 2006 survey. Error bars represent the associated confidence intervals

In 2006, it can be seen that EBA and membrane chromatography were the most commonly investigated alternative bioseparation techniques, which mimics the trend seen in the techniques which were actually adopted for manufacturing processes in 2006 (Figure 4.6).

These similarities can most likely be attributed to the fact that, as discussed these techniques are in principle based upon traditional downstream unit operations. As a

result engineers will be, to a certain extent, familiar with the development of these processes. This in turn makes it more likely that they would be chosen as candidates for investigation. The fact that these techniques are more widely adopted than other alternatives is therefore simply a consequence of this greater level of investigation and acceptance.

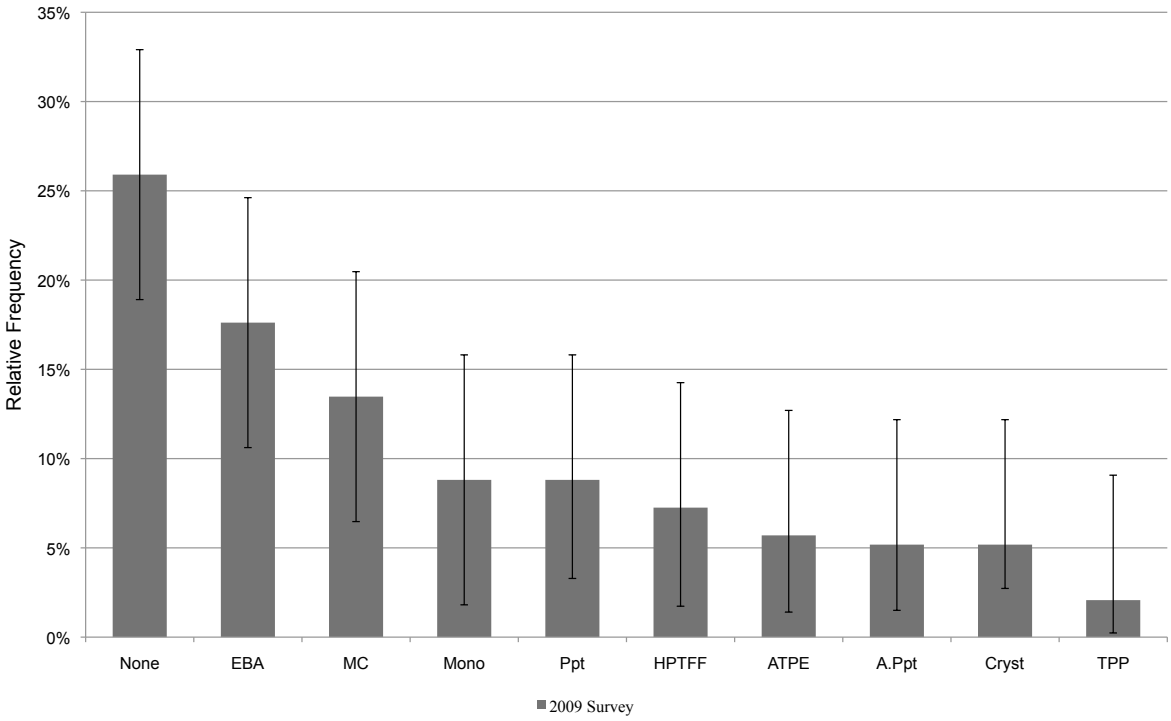


Figure 4.11: Alternative bioseparation techniques which are either being evaluated, or have been evaluated in the past by respondents to the 2009 survey. Error bars represent the associated confidence intervals

Moving to 2009, Figure 4.11 shows the alternative techniques which have been evaluated by respondents to the 2009 survey. From this it can be seen that the investigation and evaluation of alternative bioseparation techniques is no longer narrowly focused on just EBA and membrane chromatography, but has instead broadened to encompass a wider range of different techniques, including in particular precipitation processes. This again reflects the trend seen in the bioseparation techniques, which are actually adopted for large scale biomanufacturing (Figure 4.7) in 2009.

Information on the techniques which have been most widely investigated is useful as it provides insight into the those which are most attractive to process engineers.

However of particular interest, are the reasons for these alternative techniques being disregarded for use. It may be the case that some of these techniques have fundamental limitations, which make them unsuitable for use at scale. Alternatively, techniques may only be suitable for very specific processing scenarios. In order to gain an impression of the current limitations of these alternative techniques and the major factors hindering their adoption into large scale manufacturing processes, respondents were asked to give reasons why the techniques, which they had evaluated, had been subsequently disregarded. It was found from the responses given, that the reasons given could be broadly categorised into eight different groups.

A common reason given was that the alternative technique did not provide a significant or sufficient advantage, either in terms of the performance (i.e. yield, purification or throughput), or the economics, over conventional unit operations. The emergent nature of many of these technologies means that the regulatory hurdles, which must be overcome in order to integrate them into manufacturing processes, would be greater than for more conventional unit operations. New processes must be thoroughly characterised and validated before they may be used. Furthermore, from an operational standpoint, staff will need to be trained to deal with the new technology and new equipment and materials will need to be procured. In this regard, incorporation of a new separation technology into the manufacturing process is a resource draining undertaking. On top of this, there is always the risk that the new technology will not work at large scale, or that some unforeseen issues may arise. The large financial and technical risk a manufacturer will therefore expose themselves to, when looking towards utilizing an alternative technique, means that the benefits which are received in return must be sufficient so as to justify this risk. Indeed, based on the responses received, biomanufacturers generally feel that not only must an alternative technique outperform the conventional process, but it also must outperform it by a significant margin. One of the most common reasons for disregarding an alternative was that it was not able to meet this criterion.

Unsatisfactory performance was another reason cited by respondents. Many alternative techniques were found simply not to work with the feed material with which

they were challenged, providing a product of insufficient quantity or quality. The reason for this is again most likely due to the emergent nature of many of these alternative techniques, meaning either that the technology which is involved has not been sufficiently developed, or that the understanding of the mechanisms of separation are not fully understood and therefore cannot be effectively manipulated. Indeed it is most likely a combination of both of these factors.

The inability to scale-up these alternative processes was another reason given by respondents for their being disregarded for use, as was the high cost associated with their operation. As detailed previously, the economic considerations of packed bed chromatography was one of the major reasons the industry began to look towards alternative technologies. However, as some respondents indicated, whilst come alternatives may utilize cheap consumables and raw materials, in terms of specific costs, the quantity in which these are required actually resulted in the technique being more expensive to operate than the conventional counterpart.

Aside from the technical and economic aspects, the logistics of manufacturing is another area of concern, particularly with regards to a reliable supply of raw materials and consumables. This is an issue for some of these alternative techniques, which may require custom-built large-scale equipment, specialized consumables and exotic raw materials. The lack of a guaranteed supply of these resources was, according to respondents, enough to make them disregard these alternative techniques. Such reasoning may help to account for the relatively high adoption of techniques such as Membrane chromatography and HPTFF. As already discussed these techniques share similarities in terms of the mechanisms of separation with conventional unit operations. However they also have comparable materials and equipment requirements. Thus it would be feasible to operate a membrane chromatography process, using a conventional packed bed chromatography skid, as it would be similarly feasible to replace the ultrafiltration membrane in a TFF skid, with a charged HPTFF membrane. Techniques such as CSAF and TPP possess no such similarities and their attractiveness may suffer as a result.

Some respondents cited a poor fit with existing processes as a reason for disre-

garding an alternative technique. This may be particularly applicable to contract manufacturers and organisations, which utilise multi-product facilities. In such cases, it is important that new technologies may be easily integrated into the existing facility without disruption of the other processes housed within the plant.

The level of maturity of these alternatives was finally also given as a reason for their being disregarded for use. This may be taken as a catchall term encompassing many of the reasons described previously for alternative techniques not being adopted. For instance, a lack of maturity suggests that the technique is not sufficiently understood and therefore process development, scale-up and validation are all more difficult. An immature technology would also imply that the necessary equipment and materials required to operation the process are not widely available.

Figure 4.12 shows the relative frequency at which the above described reasons were cited as being responsible for the disregarding of an alternative technique, in the 2006 and 2009 surveys.

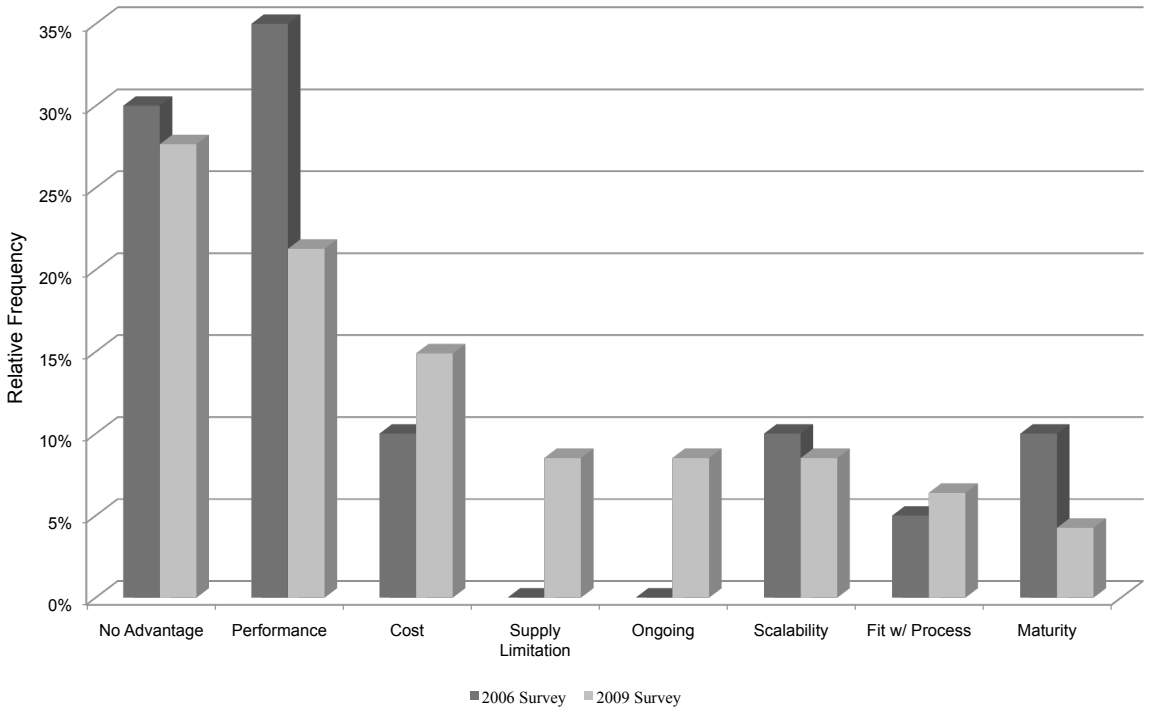


Figure 4.12: Reasons given for disregarding the use of evaluated alternative techniques

It can be seen that in both 2006 and in 2009, the most commonly cited reasons for an alternative technique being disregarded were the fact that they did not provide

a sufficient level of performance and that they offered no significant advantage over the conventional process.

Comparing the results from 2006 and those from 2009, it can be seen that the relative frequency at which performance was cited as the reason for the technique being disregarded has decreased, and instead reasons such as high cost and material and equipment supply limitations have emerged. It would seem therefore that whilst the major issues associated with these alternative techniques are still mainly technical, development of these techniques over the intervening time period has meant that at least some respondents have been able to overcome these problems. Instead the hurdles faced by the engineers looking to adopt these techniques are now either economical or logistical.

Figure 4.13 shows the relative frequency of reasons given for disregarding the use of the top five most widely investigated alternative bioseparation techniques, based on the results of the 2009 survey.

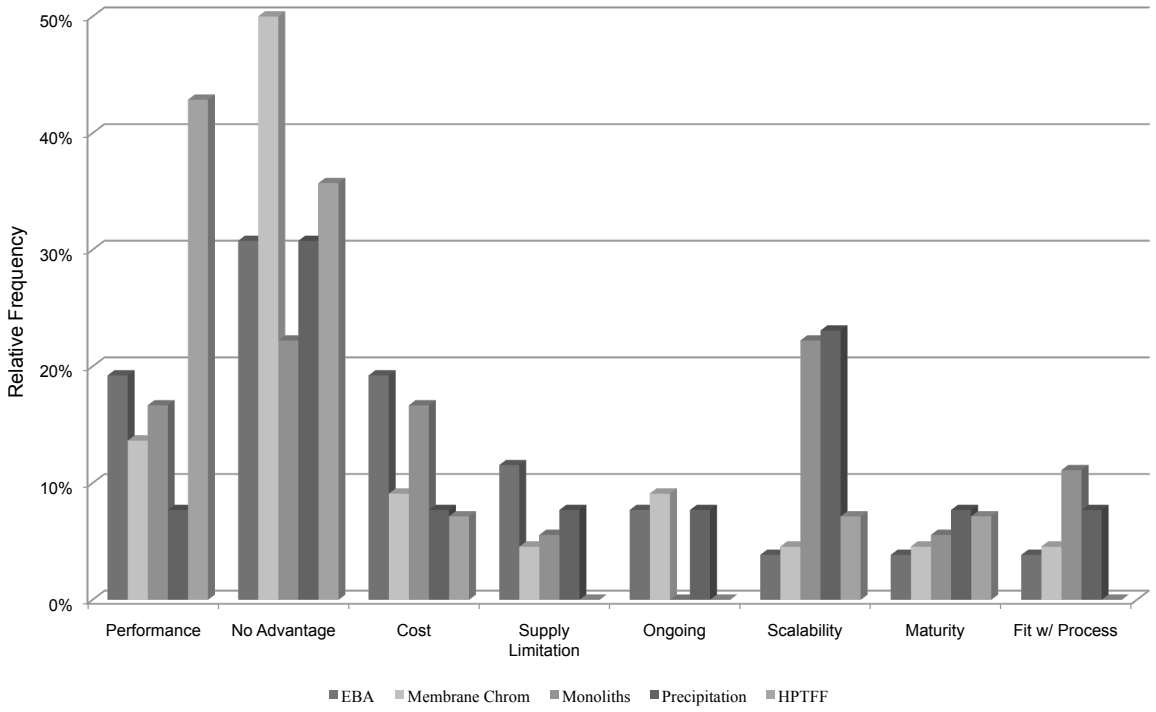


Figure 4.13: Reasons for disregarding some alternative bioseparation techniques (2009 survey)

The data presented in Figure 4.13 helps to show some of the issues commonly

encountered with these alternative techniques. Insufficient performance and a lack of an advantage over conventional techniques were commonly cited as reasons for disregarding all of these techniques. However it can also be seen, for example, that a lack of scalability was frequently given as grounds for disregarding the use of precipitation and monoliths. The scalability of monoliths is a known issue, as the synthesis of large monoliths with a consistent pore structure enabling robust chromatographic separation, is currently difficult, as was alluded to in Chapter 3. As a result large-scale monolith chromatography processes, particularly if they are to be used in bind and elute mode, would require the use of large numbers of monolith modules, which from a process economics standpoint is not a viable option. Precipitation, being a bulk mixing process, should be fairly amenable to process scale up. The fact that scalability issues were frequently encountered suggests that ensuring uniform precipitation conditions throughout the reactor at larger scales can be problematic.

The poor performance of HPTFF processes was commonly given as the reason for disregarding this alternative technique, with several respondents describing how they encountered issues with product precipitation during the diafiltration process. HPTFF processes need to be run at low ionic strength conditions, particularly if charged membranes are used, in order to achieve the best purification performance.[64] However such conditions may also reduce the solubility of some proteins, which may explain the precipitation issues encountered. Since the use of low ionic strength is a key operating constraint on HPTFF processes, such issues point to an incompatibility between the product and the technique.

4.4.8 Future Challenges

The interest in the use of alternative bioseparation techniques arose originally, close to the start of the century, as a response to concerns regarding the potential capacity limitations associated with packed bed chromatography used in mAb manufacturing. These concerns arose due to the increases in cell culture titre, which were being seen upstream.

Since then opinions on the matter have been split. There are some who have

argued that there is an absolute need to investigate and adopt alternative processing strategies, moving away from the currently established purification platform used for the manufacture of mAb products. This group has generally promoted more radical departures from the conventional, investigating separation techniques such as precipitation and the use of aqueous two-phase systems.

Others have argued that whilst packed bed chromatographic steps, and in particular protein A do indeed place limitations upon the productivity of a manufacturing facility, the current levels of demand for mAb products are such that these constraints are no where close to being challenged. Thus whilst the downstream purification train of a production facility may not be able to handle 100kg of mAb in a single batch without compromising overall process productivity, it is not actually necessary to manufacture this much material.

Finally some feel that the multi-ton manufacturing of mAb products will not become a reality, and as such the concerns regarding the capacity constraints of Protein A chromatography are moot. Instead, as more monoclonal antibody products emerge from development pipelines, companies will find their products competing in a crowded market for similar indications. As such, these organisations will be in a situation where rather than producing vast quantities of a single blockbuster drug, they will need to manufacture smaller quantities of a much wider range of products. Such a situation will necessitate the use of multi-product facilities, utilising flexible process platforms.

In the face of this range of opinions it was felt that it would be interesting to determine exactly which viewpoints were the most widely held in the industry. The final section of the 2009 survey, asked respondents what they felt was the major challenge now being faced by biomanufacturing. Respondents were asked firstly whether they felt that the biggest issue, which manufacturers would face, would be the challenges of multi-ton manufacturing, the problems associated with the design and operation of multi-product facilities or if it would be a combination of both. They were then asked to identify the ways in which, they felt, would be the best to deal with these challenges, whatever they may be.

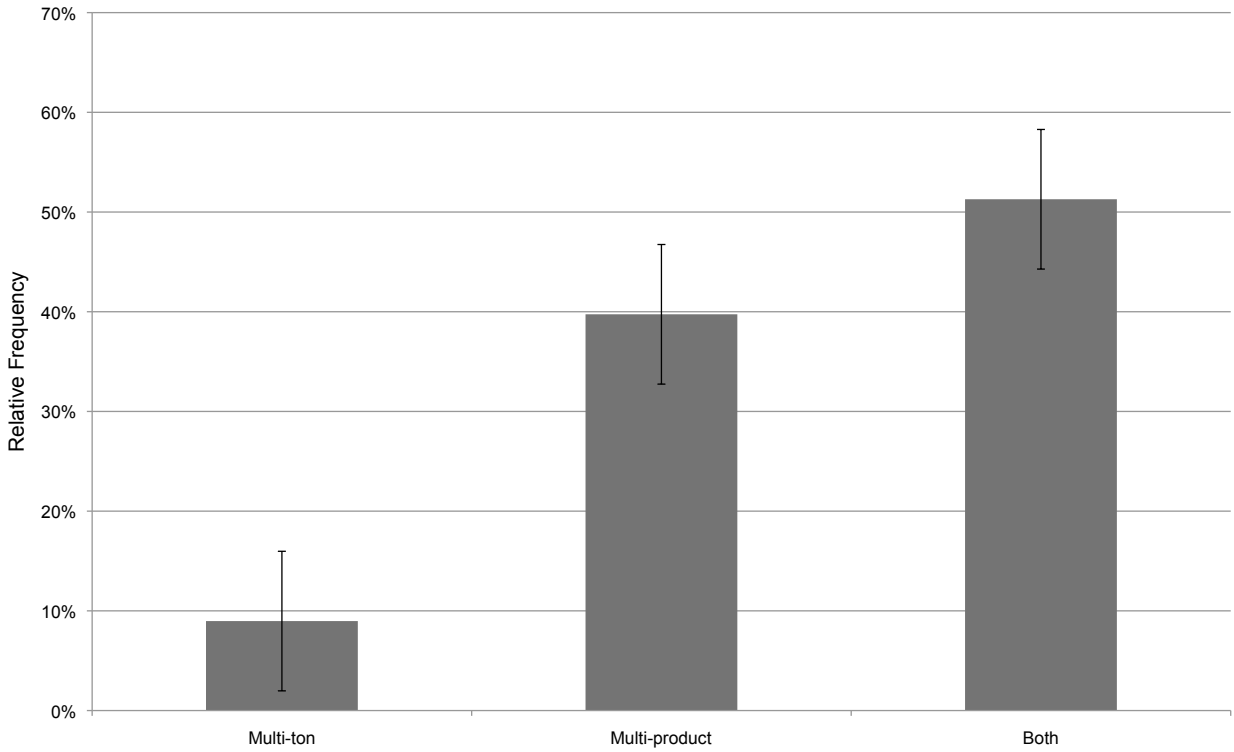


Figure 4.14: Major challenges currently faced by biomanufacturing according to respondents to 2009 survey. Error bars represent the associated confidence intervals

Figure 4.14 shows that most survey respondents felt that both multi-ton and multi-product manufacturing will pose significant challenges to bioprocess engineers in the near future. It can also be seen that very few respondents feel that multi-ton production will be the lone challenge faced by the industry. Instead almost 4 times as many respondents felt that the development and operation of multi-product facilities will be the major concern. Thus from this it seems that whilst the majority of engineers feel that both multi-ton and multi-product manufacturing will pose some challenges in the future of biomanufacturing, of the two, multi-product manufacturing will present the more overriding issue.

Respondents were then asked what they felt was the best way of dealing with these upcoming challenges, either choosing from a list of pre-defined options, or describing their own suggestion. The pre-defined list contained a number of options reflecting ways in which those within industry have suggested these challenges may be addressed. Figure 4.15 shows the processing options which the survey respondents felt were the most suitable to deal with future challenges to the industry.

From Figure 4.15, it can be seen that the use of disposables was one of the two most

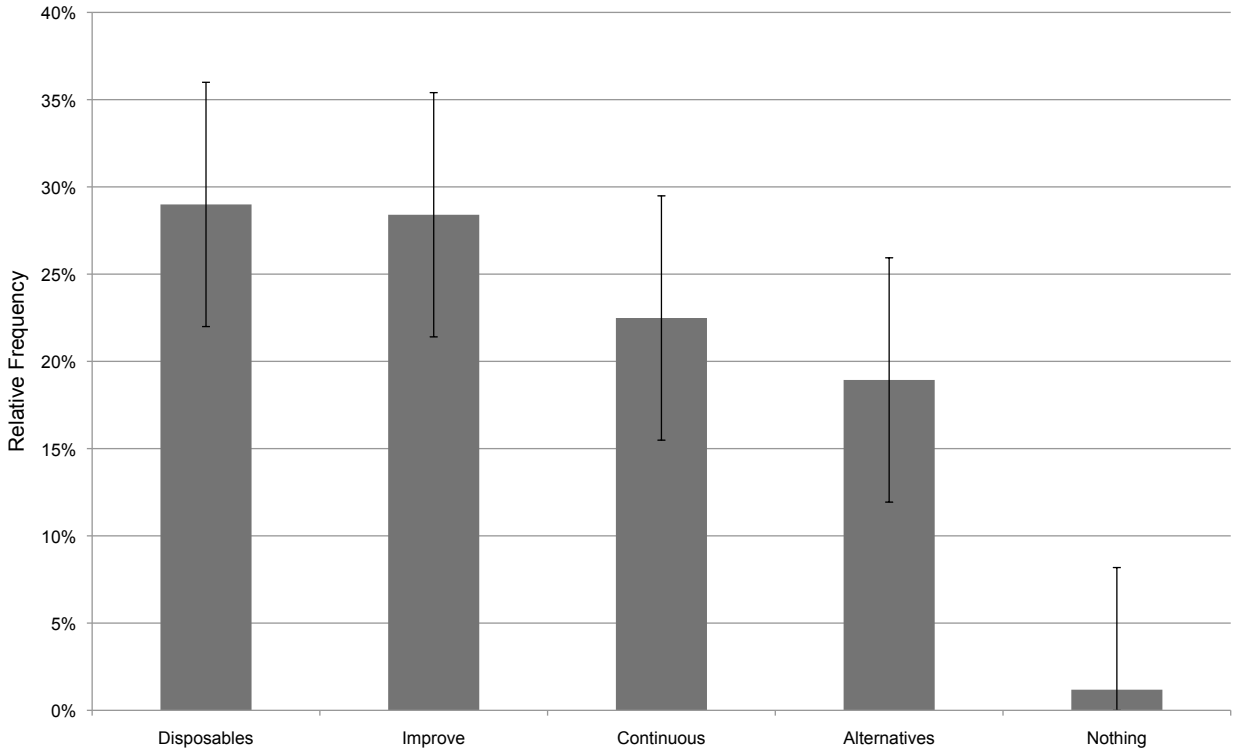


Figure 4.15: Most appropriate ways of addressing upcoming challenges in biomanufacturing according to respondents to 2009 survey. Error bars represent the associated confidence intervals

popular choices. This is most likely linked to the high proportion of respondents who felt that the major challenge for biomanufacturing would be the use of multi-product facilities. Disposable unit operations are ideally suited for use in such facilities as they allow for more rapid changeovers between manufacturing campaigns, which in turn enable higher facility utilisation and increased overall plant productivities.

Of a similar level of popularity, is the option of improving current technologies. A significant proportion within the industry clearly feel that the best way of increasing process productivity, whether it be for multi-ton or multi-product manufacturing, is through the development of conventional unit operations rather than through more radical means such as the adoption of alternative separation technologies, thereby favouring evolution over revolution. An example of this would be the development of Protein A chromatography resins with higher binding capacities, which can be operated at faster flow rates. Another example would be ultrafiltration membranes with more uniform pore sizes and thus tighter molecular weight cut-offs allowing for

higher process yields. Such an approach is popular, as it would have the minimum impact upon existing processes and facilities.

The use of continuous processing and adoption of alternative bioseparation techniques are arguably, equally attractive options, both being less attractive than the use of disposable technologies and the improvement of current technologies.

Continuous processing through the use of for example simulated moving bed chromatography to enable continuous Protein A capture would therefore increase process productivity. Such an approach would maximise the efficiency of each process step at the expense of increased operational complexity, which as was discussed previously, is a process attribute upon which process engineers place a relatively high level of importance, possibly explaining the lower attractiveness of this particular option.

Finally, fewer than 20% of respondents felt that the use of alternative bioseparation techniques was the most suitable option for tackling the future challenges in biomanufacturing. This is fairly understandable considering the fact that most respondents felt that multi-product manufacturing is where the major challenges will come from in the near future. Effective operation of a multi-product facility would ideally involve the use of a process platform. This process template, whilst not an entirely generic process, would contain a common set of unit operations, arranged into an efficient sequence allowing for the processing of the entire range of products which are to be manufactured within the multi-product facility. Many of the alternative bioseparation techniques do not currently lend themselves well to this platform approach. Unlike Protein A affinity chromatography which relies on ligand-substrate interactions common to all IgG molecules, techniques such as precipitation rely on very specific protein solubility characteristics in order to effect bioseparation. As a result, whilst a precipitation process may enable purification of Antibody A, it will not necessarily work for Antibody B.

4.5 Conclusions

The aim of this study was to gain insights into the level to which alternative technologies had permeated into current biomanufacturing processes. From the results obtained a number of conclusions may be drawn. Firstly it is clear from the results of this survey that alternative bioseparation techniques have entered the collective consciousness of the Biopharmaceutical industry. Based on the high percentage of respondents who indicated at least the investigation of alternative techniques, it is clear that people are at any rate considering the use of alternative bioseparation techniques in their manufacturing processes. Comparing the results between 2006 and 2009, the increase in interest in the use of alternative technologies was clearly seen, with the percentage of respondents that indicated they had investigated the use of at least one alternative bioseparation technique increasing from 65% up to 80%. A similar trend was seen in the number of respondents who stated that they used at least one alternative bioseparation technique in their process. Indeed the results of the 2009 survey show that over half of current biomanufacturing processes, it is assumed for products currently on the market or are in late stages of development, contain at least one alternative bioseparation technique. Furthermore, the range of techniques, which either are being investigated or have been incorporated into manufacturing processes has expanded. Thus it would seem that biomanufacturers are fully embracing the potential benefits of some of these technologies.

However, based on the techniques that show the highest level of investigation as well as adoption, it would seem that manufacturers are taking a fairly low risk approach towards the utilisation of these alternatives. The most popular alternative techniques represent quite a mixed group, both in terms of the mechanisms of separation utilised and their point of application within the purification train. However they all have properties, which help mitigate the risks, associated with their adoption, whether it be a high level of comparability to conventional unit operations, maturity and understanding of the technology, or precedence of utilisation. In addition, utilisation of these alternatives does not seem to have caused a dramatic shift in current

manufacturing paradigms, with the average number of conventional packed bed chromatography steps remaining constant, regardless of whether alternatives are used or not. It would seem therefore that rather than being used to replace chromatography, some of these techniques are being used to simply augment the purification process.

The reluctance to move away from conventional processing platforms is evidenced by the fact that the use of alternative bioseparation techniques is, according to survey respondents, the least attractive option when it comes to improving process productivity and facility utilisation, with the use of disposables, the improvement of existing technologies and the application of continuous processing which is the ultimate extension of process intensification, all being more desirable avenues for development. Despite this however, the trends between 2006 and 2009 show that alternative technologies is a growing area of interest amongst biomanufacturers. It is unrealistic to expect a sudden and dramatic change in the current biomanufacturing landscape, with a paradigm shift towards chromatography-free processes. Aside from the associated regulatory issues, the necessity of such a drastic change is questionable. Current downstream processing strategies are proving capable of accommodating the increases in product titres seen to date. Whether this can be sustained if the predicted increases in upstream titre are realised in the future remains open to question. However at the present time there is probably insufficient incentive to force process engineers to look at pushing for a paradigm shift because firstly it does not yet seem an imperative and secondly because doing so would carry a significant level of financial and technical risk.

However the increased level of interest of in these techniques would imply that, whilst it is not the most desirable option, engineers are actively looking for opportunities to exploit the benefits bestowed by some of these alternatives, and incorporating them into newer processes. As a result, the permeation of alternative techniques into purification processes is likely to be much more gradual. As more of these alternatives are investigated, and as their technology matures, the range of techniques being adopted into processes will widen and the frequency at which it occurs will increase. Thus rather than seeing a dramatic paradigm shift, much more likely is a paradigm

evolution, with current manufacturing platforms transforming to incorporate these alternative techniques.

A key component of this bioseparation survey was the section asking respondents to quantify the level of value placed on different process attributes such as yield, purification factor and throughput. As was revealed in Chapter 3, none of the alternative bioseparation techniques are total solutions to the challenges faced by downstream processing, in light of the advances seen upstream. Instead whilst some techniques may indeed be capable of addressing the potential cost and productivity issues associated with packed bed chromatography, they also have deficiencies in other areas such as scalability and ease of process development. A quantitative analysis of the attractiveness of these techniques for use in biomanufacturing must therefore be able to take account of these trade-offs between the inherent strengths and weaknesses of each technique. Using the data obtained from reviewing these different technologies, outlined in Chapter 3, and the importance values obtained from the survey described in this chapter, it was possible to develop a tool which may be used to perform this analysis, as shall be described in Chapter 5.

Chapter 5

A Methodology for the Comparative Evaluation of Alternative Bioseparation Technologies

5.1 Abstract

Advances in upstream technologies and growing commercial demand have led to cell culture processes of ever larger volumes and expressing at higher product titres. This has increased the burden on downstream processing. Concerns regarding the capacity limitations of packed bed chromatography have led process engineers to begin investigating new bioseparation techniques that may be considered as "alternatives" to chromatography and which could potentially offer higher processing capacities but at a lower cost. With the wide range of alternatives which are currently available, each with their own strengths and inherent limitations, coupled with the time pressures associated with process development, the challenge for process engineers is to determine which technologies are most worth investigating. This chapter presents a methodology based on a Multi-Attribute Decision Making (MADM) analysis approach, utilising both quantitative and qualitative data, which can be used to determine the "indus-

trial attractiveness" of bioseparation technologies, accounting for trade-offs between their strengths and weaknesses. By including packed bed chromatography in the analysis as a reference point, it was possible to determine the alternatives which show the most promise for use in large scale manufacturing processes. The results of this analysis show that whilst the majority of alternative techniques offer certain advantages over conventional packed bed chromatography their attractiveness overall means that currently none of these technologies may be considered as viable alternatives to chromatography. The methodology introduced in this study may be used to gain significant quantitative insight as to the key areas in which improvements are required for each technique and thus may be used as a tool to aid in further technological development.

5.2 Introduction

The results from the surveys detailed in Chapter 4 revealed a high level of interest, from within the biopharmaceutical manufacturing industry, in the use of alternative bioseparation techniques. Comparison of the results over the course of a two year time period also revealed that this level of interest has increased. It can therefore be said that engineers are at least entertaining the idea of potentially adopting some of these techniques into their manufacturing processes. The difficulty for engineers would seem to be deciding which alternatives are most worthwhile investigating. Based on the survey results the most popular alternative techniques being either utilised or evaluated are membrane chromatography and high performance tangential flow filtration. Both of these techniques could be considered to be "safe" choices, since as previously discussed, being based on established downstream separation technologies, the perceived risks associated with their adoption is considerably reduced when compared to less conventional techniques such as aqueous two phase extraction and three phase partitioning. Such tendencies are not surprising. Process development is a time-consuming endeavour, taking on average between 1 and 2 years.[103] Given the increasing pressures on speed to market, process engineers are unlikely to devote precious resources

to investigating "novel" bioseparation techniques unless there is a high probability of a beneficial return. This is exacerbated by the fact that no current alternative bioseparation techniques possess characteristics which comprehensively meet the demands placed upon downstream processing by upstream advances. For example a certain technique may be able to overcome the capacity limitations faced by packed bed chromatography, but does not perform well at large scale. Alternatively a process may be amenable to scale-up but face issues of robustness and ease of process control. When determining which techniques to investigate a process engineer must be able to account for such trade-offs between different process characteristics.

Given the number of decision making factors which need to be accounted for and the relatively limited level of understanding of many of these alternative techniques, it is not surprising that the first selection criteria used by many engineers in determining which alternative to investigate, is the level of perceived risk associated with these techniques, which in most cases is a function of a range of parameters such as the maturity of the technology, the experience of the engineer and attitudes within the organisation for which the engineer works. The danger would be that these subjective parameters are masking the "industrial applicability" of these alternative techniques and their potential for being true alternatives to packed bed chromatography. The risk then is that alternatives with genuine potential are not being investigated because decisions on which techniques to evaluate are based on subjective criteria. Instead these decisions should be based on a completely rational and quantitative evaluation of these alternative techniques, something which has yet to be performed.

Past work has mainly concentrated on characterising the performance of the different technologies, outlining the strengths and weaknesses of each. A limited number of studies evaluate the industrial applicability of these alternative techniques with relation to one another and also to packed bed chromatography.[11, 29, 104] These studies offer some qualitative assessments of the suitability of alternative technologies for use in large scale manufacturing processes, however so far no work has been done to quantify how close these techniques are to becoming viable alternatives to packed bed chromatography. The "attractiveness" of a technique will depend upon a num-

ber of process characteristics and a trade-off between the desirable and undesirable attributes. Some of these attributes, such as for example, the ease of scale-up or ease of process development, may be difficult to quantify.

The aim of this present study was to develop a methodology which would allow the rapid identification of alternative bioseparation techniques which show the most potential for use in large scale biomanufacturing, taking into account a wide range of process characteristics including performance, scalability, ease of operation, ease of process development and process economics, and also how these characteristics trade-off against one another. By utilising both quantitative and qualitative data on each of the alternative techniques, it was intended that a purely rational analysis could be performed. The results of such an analysis would then allow a quantitative determination of the "industrial attractiveness" or "applicability" of alternatives for use in large scale biomanufacturing processes.

There are a number of different quantitative decision making tools currently used by businesses to evaluate possible alternative strategies. Those routinely used in commercial decision making include simple techniques such as Pareto Analysis and Paired Comparison Analysis, and also more powerful techniques such as Decision Trees, Plus/Minus/Interesting (PMI) and Force Field and Cost/Benefit Analysis.[105] These techniques will usually be used by commercial organisations when a set of options for changes to a business exist from which one action is to be selected. These decision making tools may then be used to determine the optimal choice to make and then also whether the change is worth making at all. The limitation of techniques such as Pareto Analysis and Paired Comparison Analysis is that they will only allow comparisons to be made between different options in terms of a single chosen metric. The aim of this study was to develop a tool which could account for trade-offs between a number of different process characteristics. Decision Trees bring in an additional level of detail, in that they account for the probability of certain outcomes which may result from making certain choices, however again, the limitation is that comparisons are made based on a single metric. Techniques such as PMI, Force Field Analysis and Cost/Benefit Analysis differ slightly in that they are mainly used once a choice

has already been made. These techniques will then usually be utilised to determine whether the chosen option is worth making at all. In the context of this study, these tools would most likely be used once an alternative separation technique has already been chosen, and an organisation wants to determine whether the benefits of incorporating the chosen technique outweigh the associated costs of adoption. As a result none of these techniques are suitable for the purposes of this study. Instead a Multi-Attribute Decision Making Analysis (MADM) approach was taken.[106, 107] MADM is a statistical technique which utilises both quantitative and qualitative data to determine the optimal solution from a range of options based on a set of different and possibly conflicting criteria, making it ideally suited to the purposes of this analysis. In this study, the optimal solution would be the bioseparation technique which has the highest level of "industrial attractiveness", and the criteria used for determining the best option would be based on attributes chosen to reflect the process characteristics detailed previously. Packed bed chromatography (both affinity based and also non-specific) was used as a reference point. By comparing the attractiveness of the alternatives to that of chromatography, the significance of the advantages offered could be determined. The list of techniques evaluated in this study was based on the bioseparation techniques outlined by Przybycien et al. (2004) in their review of alternative bioseparation technologies and have also been discussed previously in (Chapter 3). The full list of techniques which have been considered is shown in Table 5.1.

It should be noted that the list of techniques evaluated in this study was not designed to be comprehensive and that there are a number of alternative bioseparation technologies (e.g. smart polymers, synthetic protein and chemical ligands), or variations of those listed (e.g. ion exchange membrane chromatography), which are currently available but have not been included. The primary aim of this study was to develop a decision making framework which could be used to evaluate the industrial applicability of a given bioseparation technique, rather than perform a complete analysis of all available techniques. As such the techniques included were chosen to represent a wide range of operations, utilizing a range of different mechanisms for

Table 5.1: Bioseparation techniques which were evaluated using the approach developed in this study.
List of techniques taken from Przbycien et al., 2004.[29]

Bulk Separations	Field-Based Separations	Adsorptive Separation
Aqueous Two Phase Extraction (*ATPE*)	Affinity Filtration (*Aff. Fil*)	Affinity packed bed chromatography (*Aff. Chrom*)
Bulk protein crystallisation (*Cryst*)	Controlled shear affinity filtration (*CSAF*)	Affinity expanded bed adsorption chromatography (*EBA*)
Macroligand facililated three phase partitioning (*MLFTPP*)	High performance tangential flow filtration (*HPTFF*)	Magnetic adsorbent particles (*MAP*)
Secondary effect affinity precipitation (*SEAP*)		Affinity membrane chromatography (*MC*)
Three phase paritioning (*TPP*)		Affinity Monoliths (*Mono*)
		Non-specific packed bed chromatography (*PBC*)

Abbreviations for each technique, as used in the main text of the paper, are shown in *italics*

separation, in order to demonstrate the applicability of the methodology. It would be equally viable to apply the framework described herein to any bioseparation technique.

In order to focus the scope of this study, the evaluation of the different bioseparation techniques, was conducted based on a scenario in which the techniques were used for the primary capture of a monoclonal antibody (MAb) product, generated from a 20,000L mammalian cell culture, with a titre of 5g/L.

MAb was used as a model product as it is currently the most prevalent biopharmaceutical product being manufactured. In fact, the recent increases in MAb titres which have been achieved are almost entirely responsible for giving rise to the concerns regarding the possible limitations of packed bed chromatography and the resultant interest in potential alternative manufacturing strategies. It was for these reasons that MAbs were deemed to be a particularly apt model purification target for use in this study. As a consequence, Protein A was chosen as the affinity ligand used by all techniques which base their mechanisms of separation upon specific affinity interactions (Table 5.1)

Whilst the evaluation performed in this study was based upon the above context, it would be equally feasible to utilize the framework described herein to any number of processing scenarios (e.g. Intermediate Purification, Polishing, Viral clearance etc.). Again the aim of this study was to develop and show the applicability of this decision making framework for a particular set of process options for a specific purpose within

the process train, thereby demonstrating the usefulness of this tool. The objective was not to perform a comprehensive evaluation of alternative bioseparation techniques in all possible processing circumstances. Sufficient details have therefore been given to enable a user to tailor the framework in order to apply it to any desired process scenario and set of techniques.

5.3 Materials and Methods

5.3.1 Multi-Attribute Decision Making (MADM)

A weighted additive method was used to perform the MADM analysis as this is the simplest selection method.[107] The basic components of such a MADM analysis are summarised in Table 5.2, which shows a generic example of the way in which it may be performed. A MADM analysis may be considered as a matrix of dimensions i x j, where i represents the attributes and j, represents the available options. In this scenario, the objective is to determine out of two options; A and B, which is superior, with the decision being based on n number of different attributes. It should be noted that the MADM analysis can be extended to include any number of different options and is not limited to comparing only two as is the case in this example.

Table 5.2: Outline of the basic components of a MADM analysis and how options are ranked using a weighted additive method

		Options (j)	
	Importance Weight (w_i)	Option A (e.g. ATPE)	Option B (e.g. HPTFF)
Attribute 1 (e.g. Yield)	w_1	r_{1A}	r_{1B}
Attribute 2 (e.g. Purification Factor)	w_2	r_{2A}	r_{2B}
Attribute 3 (e.g. Process Cost)	w_3	r_{3A}	r_{3B}
.	.	.	.
.	.	.	.
.	.	.	.
Attribute n	w_n	r_{nA}	r_{nB}
		$S_A = \sum_i^n w_i r_{iA}$	$S_B = \sum_i^n w_i r_{iB}$

The first step, in the MADM approach, is to allocate weightings (w_i) to each of the

attributes. These weightings are numerical values which reflect the level of importance associated with each of the attributes and their impact upon determining the superior solution. The next step is to assign ratings (r_{ij}) for each of the options in each of the attributes, denoted. These must be chosen so as to reflect the characteristics of Option A and B in terms of each of the attributes used. The overall score (S_j) for Option A and B may then be calculated as shown in Equation 5.1

$$S_j = \sum_{i}^{n} w_i r_{ij} \tag{5.1}$$

Weighting and Attribute Rating Normalisation. An important aspect when performing a MADM analysis is that the weightings and ratings be normalised based on the array of observed values seen for each attribute, across the range of options. The values of w_i and r_{ij} shown in Equation 5.1 are therefore normalised values which can be described mathematically by Equations 5.2 and 5.3.

$$w_i = \frac{y_i}{\left[\sum_{i}^{n} y_i\right]} \tag{5.2}$$

$$r_{ij} = \left| \frac{x_{ij} - x_{i,worst}}{x_{i,best} - x_{i,worst}} \right| \tag{5.3}$$

Where y_i is the weighting given to attribute i and $x_{i,worst}$ and $x_{i,best}$ are the worst and best ratings for attribute i, respectively, based upon the range of ratings (x_{ij}) seen across all available options. This normalisation allows MADM to deal with both quantitative and qualitative data, since all options receive a final normalised weightings (w_i) of between 0 and 1 and ratings (r_{ij}) of between 0 and 100 for each attribute.

5.3.2 Attributes

In this study the attributes were broken down into five groups with each containing 5 to 7 attributes (see Table 5.3). In choosing the attributes, effort was made to ensure that a broad range of process characteristics, which may influence the"industrial

attractiveness" or applicability of a given bioseparation technique for use in large scale biomanufacturing, were included. Care was also taken to ensure the selected attributes did not introduce bias.

Table 5.3: Summary of the Attributes and Attribute Groups Used for the MADM Analysis

Attributes				
Performance*	**Ease of Scale-Up***	**Process Economy***	**Ease of Operation***	**Ease of Process Development***
Capacity	Scale Dependent Parameters	Consumables Cost	*Ease of Control PAT*	Ease of Validation
Concentrating Power	*Availability of Large Scale Equipment*	Equipment Costs	Handling Requirments	*Applicability of Scale Down Models*
Purification Factor	*Established Scale-Up Rules*	FCI	Labour Requirements	*Availability of Generic Process Parameters*
Resolving Power	*Precedence of Large Scale Operation*	Labour Costs	*Robustness*	*Low QC Effort*
Productivity	-	Raw Materials Cost	*Use of Organic Solvents*	*Understanding of Performance Parameters*
Yield	-	Space-Volume Efficiency	-	*Use of Platform Technology*
-	-	Utilities Cost	-	-

Qualitative attributes are shown in Italics.

*Indicates attribute group.

Process Performance. Process performance refers to the technical performance of the technique and includes attributes of process yield, purification factors and resolving power; highly important process attributes, particularly with regards to techniques being used for the manufacture of biopharmaceuticals destined for use as human therapeutics. The concentrating power of a technique was deemed to be an important process attribute, since reductions in process volume can go a long way towards reducing overall process costs (both capital and operating expenditure). Process capacity and productivity attributes were included in the analysis in order to evaluate fully the relative advantages of these alternatives over packed bed chromatography.

Ease of Operation. The ease of operation group contains attributes which reflected how easy a given technique is to operate at large scale and includes attributes of robustness and ease of control. The level of handling and labour were also included as certain alternative bioseparation techniques require extensive transfer of materials into multiple unit operations and this may be a drawback. The use of organic solvents by a technique may also be seen as a shortcoming since the use of such components

requires special safety measures which in turn can have a deleterious impact upon the ease of operation.

Process Economics. This particular attribute group mainly concentrated on the various cost elements associated with the set-up and operation of a bioseparation technique. Attributes such as the equipment costs and the fixed capital investment were included such that the MADM analysis would reflect the costs of actually setting up a bioseparation technique. This was considered to be particularly important since some alternatives may be relatively capital intensive. Cost components such as raw materials and consumables were included in order to account for alternative techniques with relatively low raw material and consumable requirements.

Labour costs were included to reflect the fact that techniques comprising multiple steps may require a larger labour force to operate whilst techniques which may be relatively simple to operate, but have long cycle times, will also increase the labour costs.

The Space Volume efficiency is essentially a measure of the volume of equipment required to process a certain volume of feed. This ultimately would impact to a certain extent upon the Fixed Capital Investment, since techniques which have a low Space-Volume efficiency would require a larger plant to house the process. This attribute reflects how well a bioseparation technique will fit with existing facilities. Such an attribute would be of a high importance to organisations such as contract manufacturers where a certain amount of plant space will be available for a downstream process. Techniques with large foot-prints will therefore be unattractive to such organisations and this attribute can be used to reflect this.

Scalability. Attributes included in this category were chosen to reflect how easy a bioseparation technique is to scale-up. Precedence of large scale operation was considered to be a particularly important attribute, since if a technique has been operated successfully at a large scale in the past, then the probability of achieving this again would be significantly increased. This attribute accounts for the fact that some alternative techniques, whilst showing promising results at a small scale, have not been reported operating at large scale.

The availability of established scale-up rules and also the number of scale dependent critical process parameters are both attributes directly linked to how easy a process is to scale up. If established scale-up rules exist for a particular technique, which have been effectively utilised in the past, then this would significantly increase the ease of scale-up. Along with this the lower the number of critical scale dependent parameters the easier it will be to increase process scale without compromising process performance. The availability of large scale equipment was chosen as an attribute to reflect the ease of scale-up of a process. If equipment is readily available from suppliers, this removes the need for custom manufacture.

Ease of Process Development. Process development is a time consuming process and so the ease of process development for a technique was considered an important aspect when evaluating different bioseparation technologies. The level of understanding of the mechanisms of separation exploited in each technique was considered important as this would impact the difficulty of process development.

The majority of process development work is done at small scale and it is therefore important that scale-down models which accurately represent the behaviour of the technique at a large scale are available. The use of established technology and the availability of generic processing conditions are two connected attributes which can impact upon the ease of process development. If a technique is based on established technology and/or generic processing conditions are available, then this will provide the process engineer a starting point from which process optimisation may be based. Validation and Quality Control (QC) are time consuming but vital procedures. Anything that can reduce the validation or quality control effort associated with a particular technique, such as a high level of process robustness or ease of process control, can significantly increase the speed of process development. The ease of validation and the QC effort were used as attributes to reflect these aspects.

5.3.3 Importance Weightings and Attribute Ratings

The final components of the MADM analysis are the importance weightings associated with each of the process attributes and also the ratings assigned to each attribute for

each of the bioseparation techniques. In order to determine the numerical values of these components, several research tools were used:

Survey Responses. The level of importance associated with each of the process attributes and attribute groups shown in Table 5.3 were determined from a survey distributed to biomanufacturers (Chapter 3). Part of this survey asked respondents to rate the level of importance placed on the attributes and attribute groups shown in Table 5.3, when considering whether or not to adopt a particular bioseparation technique. These ratings were based on a scale of 1 (representing a low level of importance) to 5 (representing a high level of importance). From this portion of the survey, a range of importance ratings were received for each of the attributes and attribute groups. Continuous probability distributions were then fitted to this data and used as the basis for the importance weightings in the MADM analysis.

Examples of the discrete frequency distributions and the fitted continuous probability functions obtained for the importance ratings of some process economics-related attributes are shown in Figure 5.1.

As would be expected, the level of importance placed on different process attributes will vary depending upon the type and size of organization, whether they are well established companies with strong drug pipelines or newer ones with relatively smaller portfolios. This variation in opinions is reflected by the distributions in importance ratings exemplified in Figure 5.1

Process Descriptions. Detailed process descriptions were generated from relevant literature for the different bioseparation technologies in order to develop the ratings of the more qualitative attributes for each of the alternatives. These describe the characteristics of the different techniques, including details of their mechanisms of separation, ease of scale-up, technological developments and also importantly, their limitations. Additionally, these descriptions were used to obtain information on certain quantitative attributes such as typical process yields and purification factors.

Process Models. Process models were developed, from available relevant literature for each of the bioseparation techniques considered. These were based on simple mass-balances and were used to determine factors including the capital and operating costs

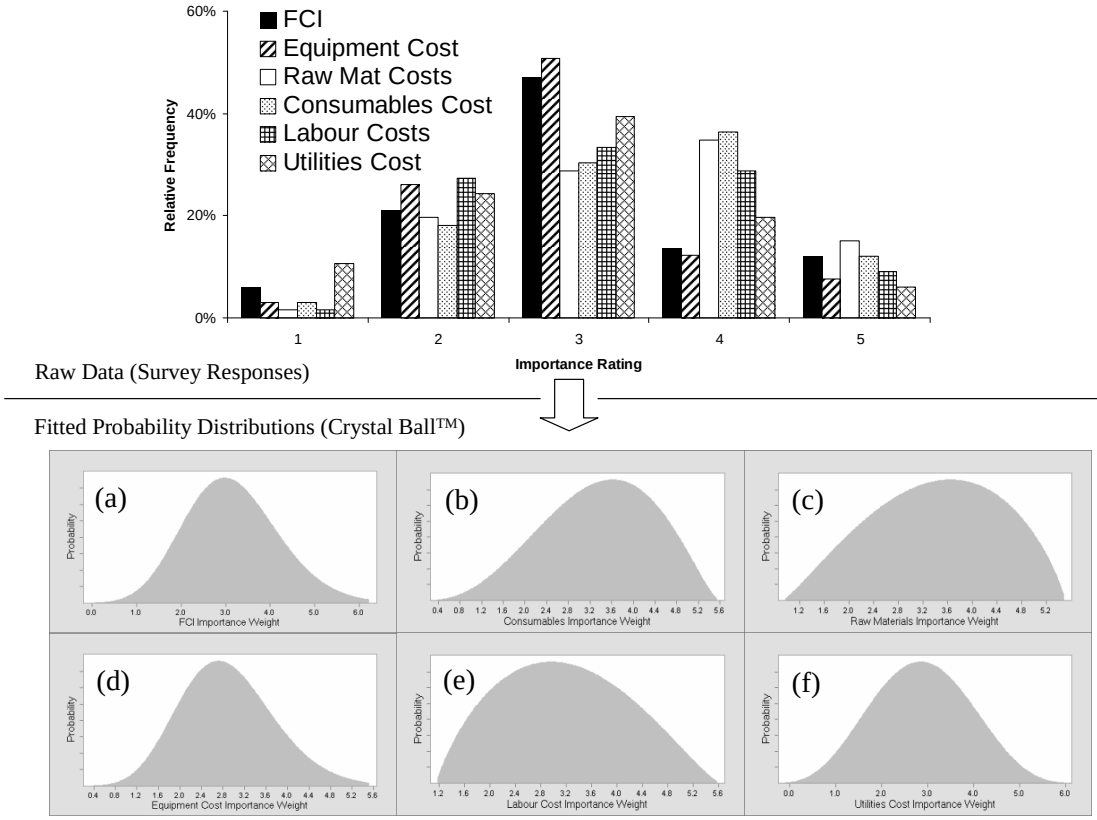


Figure 5.1: Example showing the fitting of continuous probability distributions to raw survey data on the importance ratings assigned to different attributes within the process economics attribute group.

associated with each technique as well as process characteristics such as capacity and productivity.

5.3.4 MADM Scoring Technique

The MADM analysis was performed in two discrete steps as summarised in Figure 5.2.

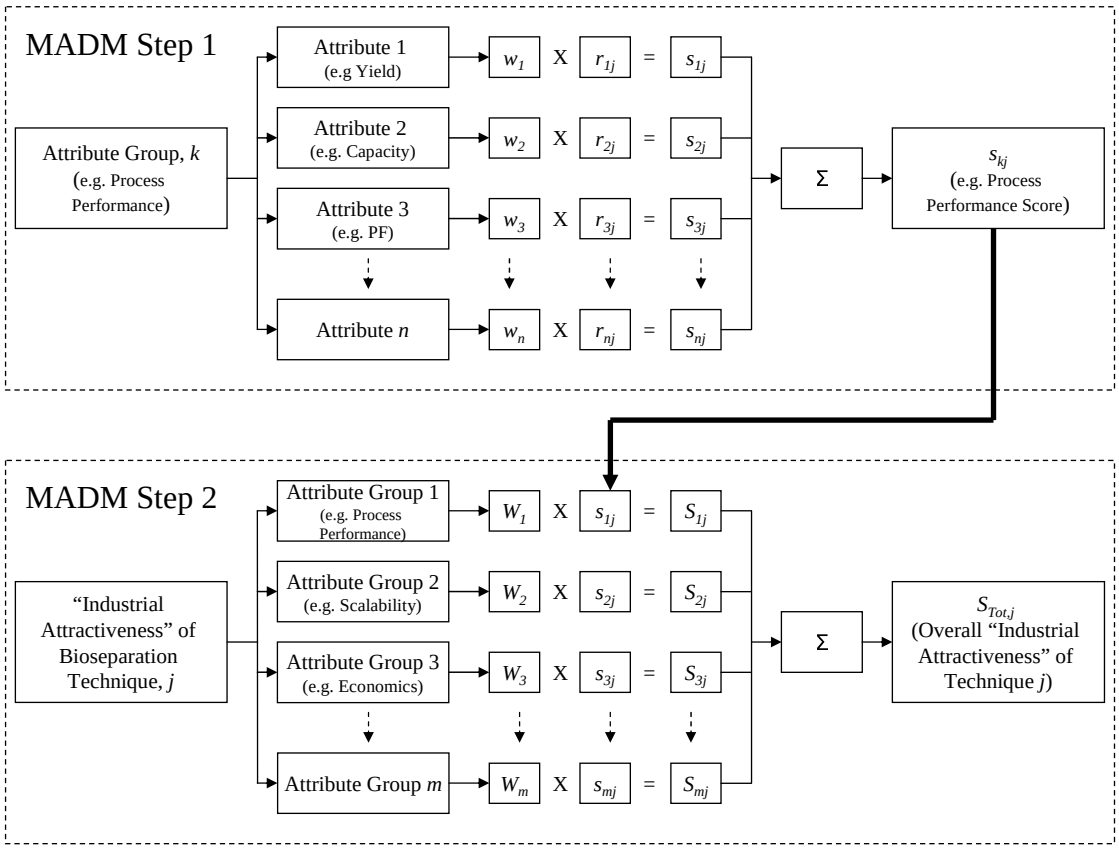


Figure 5.2: Schematic showing the flow of data for the two consecutive rounds of MADM analysis

MADM Step 1 - Attribute Group Scoring Initially techniques were scored in terms of the individual attribute groups with each receiving a score reflecting their Performance, Process Economy, Ease of Operation, Scalability and Ease of Process Development. The score obtained by a particular technique (j) for a particular attribute group (S_{kj}) can be related to the number of process attributes in each group (n), the normalised importance weighting associated with each attribute (w_i) and the normalised rating for that technique (r_{ij}) by Equation 5.4

$$S_{kj} = \sum_{i}^{n} w_i r_{ij} \tag{5.4}$$

The weightings (w_i) were obtained from analysis of the aforementioned survey (Chapter 3) whilst the ratings were obtained from both the process models (quantitative attributes) and also the process descriptions (quantitative and qualitative attributes).

MADM Step 2 - Overall "industrial attractiveness" scoring The scores obtained from Step 1 were then fed into a second round of MADM analysis (Figure 5.2), whereby the process attribute groups were each used as attributes themselves. The importance weightings (W_k) used for each of the attribute groups (k), were determined using the survey and the scores (S_{kj}) from the first round of MADM were used as the corresponding attribute ratings in order to obtain an overall score ($S_{Tot,j}$) representing the industrial attractiveness of each technique (j) (Equation 5.5).

$$S_{Tot,j} = \sum_{k}^{m} W_k S_{kj} \tag{5.5}$$

The overall attractiveness score for bioseparation alternative j can therefore be expressed by Equation 5.6.

$$S_{Tot,j} = \sum_{k}^{m} W_k \left(\sum_{i}^{n} w_i r_{ij} \right)_{kj} \tag{5.6}$$

Where n represents the number of attributes within each attribute group and m represents the overall number of attribute groups used in determining the industrial attractiveness of the techniques.

5.3.5 Monte Carlo Simulations

A key advantage of using a MADM analysis approach is the ability of MADM to handle uncertainty in the input values without sacrificing information present in the raw data. For example the importance weightings were based on the range of survey responses. As described previously, the level of importance placed on certain process

attributes by an organisation can be expected to vary depending upon its size and particular circumstances as reflected by the distributions presented in Figure 1. A certain degree of uncertainty therefore exists regarding the exact weighting given to each of the attributes. Use of an average value would sacrifice some of the information present in the raw data since clearly some respondents regard a particular attribute as being of a lower importance than the average, whilst others place higher levels of importance upon it. Similar uncertainties exist within the ratings for the techniques in each of the attribute areas. MADM allows non-deterministic analyses to be performed; such that ranges of scores for each technique may be calculated, reflecting the uncertainty present in the input data. By defining probability distributions across the ranges of importance weightings and attribute ratings (as shown in Figure 5.1), the level of uncertainty associated with each score can then be incorporated within a Monte Carlo simulation during which numbers are "randomly" selected from within these specified ranges and used to return a population of scores. By repeating this calculation procedure for a specified number of iterations (10,000 in this study), with each time different numbers being randomly selected from the appropriate ranges it is ultimately possible to compute the estimated full range of scores which the technique may obtain for a particular process attribute. The results of these Monte Carlo simulations may be presented in the form of a probability distribution histogram allowing the most likely score a technique will obtain and also the range of all possible scores to be identified. An example of such a non-deterministic analysis is shown in Figure 5.3, which displays the probability distribution of the score obtained by Crystallisation (Cryst) for the productivity attribute. In this case Crystallisation will most likely obtain a productivity score of approximately 2.5 but based on the uncertainty present in both the importance weighting associated with this attribute and also the process yields, the score may lie anywhere in the range 0.1 and 5.4.

5.3.6 Attribute Importance Weights

As described previously, continuous probability distributions were generated to describe the distribution of importance ratings assigned to different process attributes

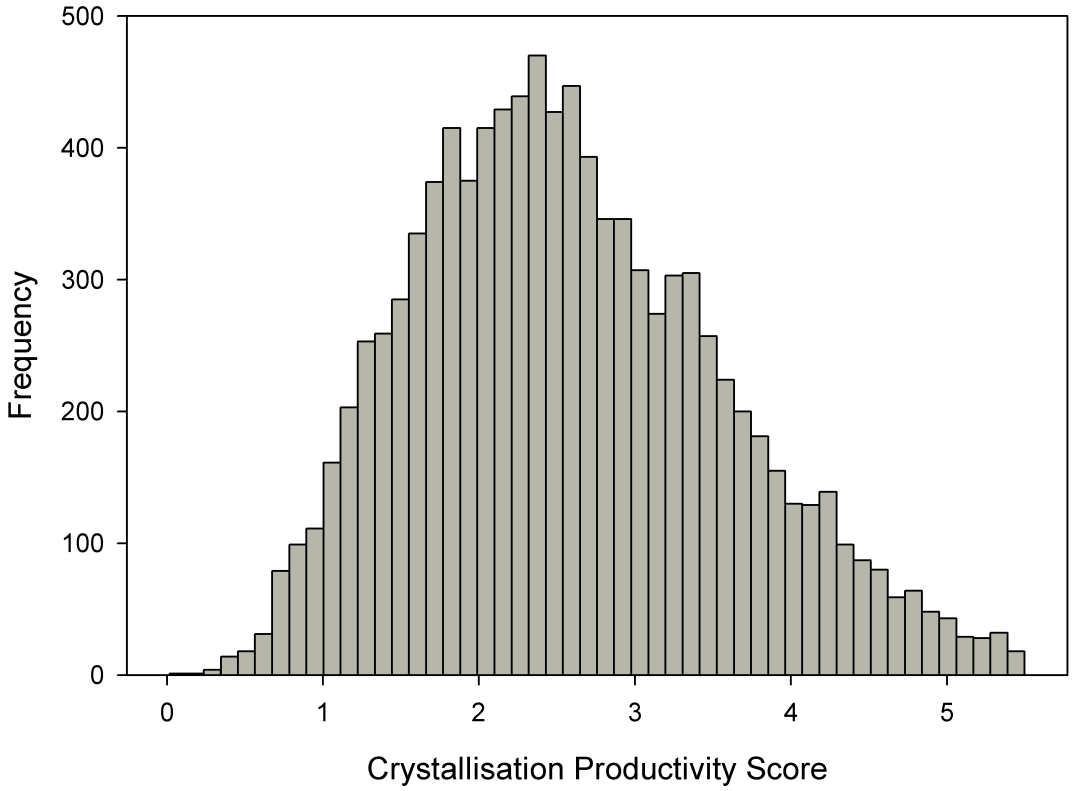


Figure 5.3: Histogram showing the range of scores crystallisation received for the productivity attribute across the range of Monte-Carlo iterations performed within the MADM analysis.

by respondents to a bioseparation survey. An example of the probability distributions defined for the importance of attributes within the process economy attribute group is shown in Table 5.4. The numbers in brackets indicate the arguments used to define each distribution.

Table 5.4: Example of the Probability Distributions Used to Describe the Level of Importance Placed Upon Individual Attributes Within the Process Economy Attribute Group and the Level of Importance Placed Upon the Group as a Whole.

Attribute Group	Attribute	Probability Distributions for Attribute Importance Weightings	Probability Distribution for Attribute Group Importance Weights
Process Economy	Fixed Capital Investment	Gamma (Loc = -3.08, Scale = 0.18, Shape = 34.64)	Beta (1.55, 5.72, 2.61, 2.36)
	Equipment Costs	Gamma (-1.48, 0.18, 24.31)	
	Raw Materials Costs	Beta (min = 0.92, max = 5.50, α = 2.21, β = 1.83)	
	Consumables Cost	Beta (0.15, 5.59, 3.56, 2.47)	
	Space-Volume Efficiency	Gamma (-3.08, 0.18, 34.64)	
	Labour Costs	Beta (1.16, 5.62, 1.87, 2.28)	
	Utilities Costs	Beta (-0.50, 6.44, 4.86, 5.17)	

5.3.7 Attribute Ratings for Techniques

Ratings for each of the techniques in each of the attribute areas were generated based on data from process descriptions, process models and also survey responses, as described previously. A combination of quantitative and qualitative attributes was used in this analysis with ratings given to each attribute, based on a consistent marking scheme. An example, showing the marking scheme used for attributes within the ease of scale-up attribute group, is shown in Table 5.5. The purpose of this scheme was to remove bias from the analysis. For ratings where a level of uncertainty existed, triangular probability distributions were defined across the range by default. A number of relationships between different quantitative attributes were involved and are summarised in Figure 5.4.

Yields and Purification Factors. The ratings used for the yield attribute for the different techniques were based on the reported yields from relevant literature. Ranges of possible yields were collected for each of the techniques and were then used to define the probability distributions for the yields achievable by each technique. The

Table 5.5: Example of the Probability Distributions Used to Describe the Level of Importance Placed Upon Individual Attributes Within the Process Economy Attribute Group and the Level of Importance Placed Upon the Group as a Whole.

Groups	Attributes	Scoring System
Ease of Scale-up	Established scale-up rules	Score based on a 0 to 100 feasible range, with a score of 0 indicating a technique, which has no established scale-up rules, a score of 25 indicates a technique for which some scale-up rules exist giving rough indications of ways in which process may be scaled up but with no firmly established protocols, a score of 50 indicating a technique for which scale-up rules exist but require trade-off and prioritisation of different process parameters, a score of 75 indicating a technique for which established scale up rules exist enabling maintenance of some critical process parameters and a score of 100 indicating a technique for which established scale-up rules exist enabling maintenance of all critical process parameters.
	Availability of large scale equipment	Score based on a 0 to 100 feasible range with a score of 0 indicating that no large scale equipment exists, and it must all be custom made, a score of 25 indicates that the technique uses a small amount of existing technology, but most of the equipment needs to be custom made, a score of 50 indicates that a technique utilises a combination of both existing technology and also some equipment which requires custom manufacturing, a score of 75 indicates that the majority of equipment used is established technology and available at a large scale, but some equipment still needs to be custom made and a score of 100 indicates that the technique is based entirely on established platform technology and large scale equipment is readily available.
	Precedence of large scale operation	Score based on a 0 to 100 feasible range with a score of 0 indicating that the technique has only been demonstrated at a lab scale, a score of 25 indicating that a technique has been demonstrated at a pilot scale, a score of 50 indicating that the technique has been validated for operation at a pilot scale under GMP, a score of 75 indicating that a technique has been validated at a production scale and used to manufacture a limited range of products, a score of 100 indicating that the technique has been operated at a large scale, under GMP to manufacture a wide range of product which are commercially available.
	Scale dependent parameters	Score based on the number of scale dependent process parameters which can affect the product quality and process performance as was determined by the process descriptions generated for this study.

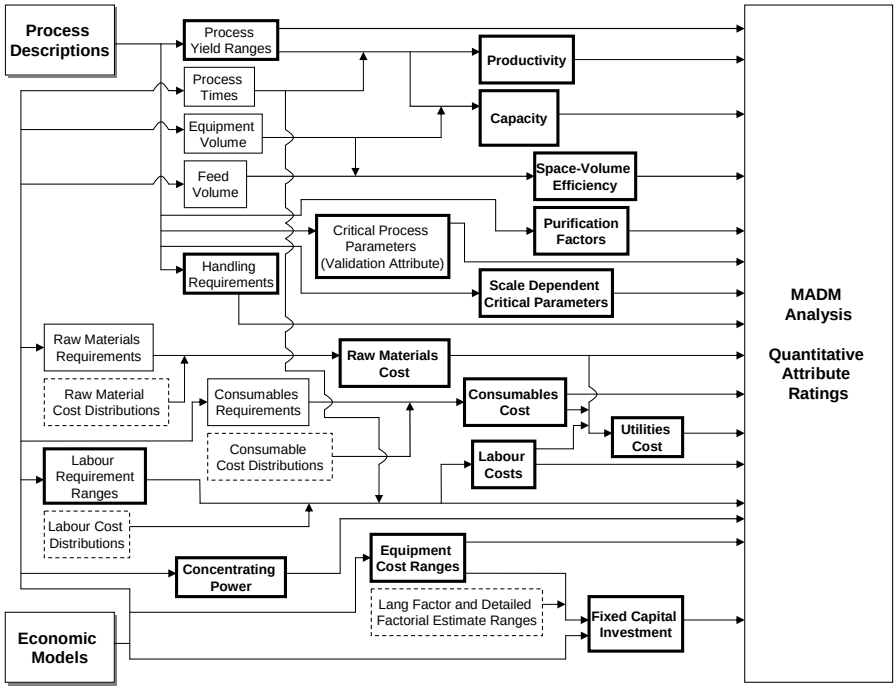


Figure 5.4: Schematic diagram showing the relationships between different quantitative process parameters and how these were fed into the MADM analysis.
Attributes directly used in the MADM analysis are indicated by thick box outlines. Parameters defined by probability distributions are indicated by dotted box outlines.

yield ranges used were based on the yields observed with each of the techniques, where possible with MAb as the target of purification. Whilst such data is readily available for affinity (Protein A) based techniques, such information is more difficult to find for some of the non-affinity based alternative bioseparation techniques. Only a limited number of studies have been performed in order to evaluate the use of these alternatives for MAb purification, most likely due to the current effectiveness of Protein A. Where studies have been performed, these are mostly of the proof of principle variety with only partial attempts made towards process optimisation. As a result, for this study, the yield ranges used for these techniques were based on those observed for a wide range of different purification targets. It was assumed that these ranges represent those which could be expected to be obtained from these techniques for a MAb target. The same was also done, using the same rationale to determine the ratings for the purification factor attribute.

Productivity and Capacity. Process productivity was based upon the process yield and durations as determined from the process models generated for this study whilst capacity was calculated based again, upon the yield and the equipment volume. Processing times were capped at 12 hours and were calculated by utilising the smallest equipment possible whilst remaining within this time constraint. The process time and associated equipment volume for each technique, as determined using the process models, was kept constant across all the Monte Carlo iterations performed whilst process yields were varied across the defined ranges as detailed previously with process productivities and capacities calculated appropriately.

The MADM calculations were set-up such that for a particular iteration for a particular technique, a common yield was used to determine the yield rating, the corresponding productivity rating and also the capacity rating.

Raw Materials and Consumables Costs. The rating each technique received for raw materials costs was based on the raw materials requirements as determined from the process models used in this study, and the cost of these raw materials. The costs of the different raw materials were defined as probability distributions based on the prices quoted by different suppliers. The MADM analysis was performed such that

the unit costs of raw materials were maintained across all techniques for any particular Monte Carlo simulation. The cost of consumables associated with each technique was determined in exactly the same manner.

Labour Costs. The labour costs associated with each technique were determined based upon the labour requirements and processing time as determined from the process models, as well as the costs of different types of labour (i.e. supervisors, operators and technicians). The process time for each technique, as described previously was maintained across all the Monte Carlo simulations. Probability distributions were defined for the amount of labour required and also the cost of this labour. These three components (process time, labour requirement and salaries) were used to calculate the overall labour costs. The MADM analysis was performed such that during a particular Monte Carlo iteration, specific labour costs were the same for all of the techniques.

Equipment Costs. Equipment costs were determined directly from the process models. Ranges were applied to these costs to reflect possible fluctuations in equipment prices from different suppliers.

Fixed Capital Investment. The Fixed Capital Investment was determined based on the equipment costs and using indices to reflect the expenditure required for installation, piping, instrumentation, construction etc. The equipment costs were defined in terms of probability distributions as described previously. The indices used to account for the additional capital expenditure were based on ranges obtained from Lang Factor and Detailed Factorial Estimates.[108]

Space-Volume Efficiency, Utilities Cost, Concentrating Power. The space volume efficiency was defined as the ratio between the feed volume and the equipment volume as determined from the process models. The cost of utilities was determined as a percentage of the overall production costs (i.e. raw materials, consumables and labour). The concentrating power of a technique was defined as the ratio between the final product concentration and the initial product concentration and was determined using the process models and also data from the process descriptions.

All distribution determinations and Monte Carlo simulations were accomplished

using the Excel Add-in; Crystal BallTM (Decisioneering, Denver, CO, USA).

5.3.8 Bioseparation Techniques

. The techniques analysed in this study (Table 5.1) were based on those detailed in previous works regarding alternative bioseparation techniques.[29, 104] Packed Bed Affinity (Protein A) Chromatography as well as Non-Specific Packed Bed Chromatography (e.g. ion exchange, hydrophobic interaction etc.) were also analysed in terms of their "industrial attractiveness" so as to provide benchmarks against which to compare the alternative separation techniques.

5.3.9 Analysis Scenario

. The fundamental aim of this study was to perform a general but fair comparison and evaluation of the different bioseparation techniques available. The MADM approach facilitates this but relies totally on the input attribute ratings used. Any bias in the input variables will be reflected in the MADM analysis. The scoring scheme used, and exemplified in Table 5.5, was devised so as to help ensure that fairness was maintained, particularly with regards to some of the more qualitative attributes. However certain quantitative attributes such as the operating costs and capital requirements will depend upon the particular processing circumstances in which the techniques are being used. Thus when generating the ratings for such attributes, care must be taken to ensure parity across the different bioseparation techniques. For example, only limited insight may be gained by comparing a given technique being used for polishing against another being used for primary capture from a crude feed stock, since inevitably different techniques will be better suited to different processing functions. It was imperative that techniques be compared under similar conditions and on a common basis. It was also important that the chosen conditions reflect those which may be commonly encountered in a current MAb production process in order to maximise the relevancy of this analysis.

To ensure parity all the techniques included in this study were analysed from

a common perspective based upon their use as primary capture steps, immediately following cell culture harvesting. Primary capture was chosen in preference to later downstream processing stages for two reasons. Firstly, process engineers are investigating the use of alternative bioseparation technologies as a direct response to the concerns being raised regarding possible capacity and productivity limitations of packed bed chromatography. MAb purification processes will normally utilise 3 chromatographic steps; Protein A affinity chromatography step allowing primary capture of the product, followed by two ion exchange steps for intermediate purification and polishing. Of these operations, a suitable candidate for replacement is the first Protein A affinity step as it is perceived as the most expensive, encounters the greatest volume of feed material and commonly exhibits low binding capacities relative to ion exchange resins.

One of the major advantages offered by many of the alternative bioseparation techniques over conventional packed bed chromatography is their ability to handle crude, solids-containing feed stocks. To reflect this, the process models for techniques not capable of handling unclarified feed streams were adjusted to include the use of a preceding centrifugation step to affect process stream clarification. The addition of this centrifugation step resulted in decreases in the yield and productivity of the techniques as well as increases in the associated capital and operating costs. Thus, within the MADM analysis, such techniques were essentially penalised for their inability to handle solids containing feeds.

A fermentation volume of 20,000L was chosen as this currently represents a typical size of mammalian cell culture for commercial MAb production and which could be purified by a single downstream processing train.[5, 102] One of the major concerns with the use of packed bed chromatography are the potential batch productivity limitations which may be encountered when processing high feed volumes due to the need for bigger columns operating over a large number of cycles. In order to address fully these concerns, the feed volumes used in the process models was set deliberately high in order to evaluate the impact of these limitations on packed bed chromatography and to examine if such limitations could be overcome by any of the alternative techniques.

Whilst MAb titres of up to 10g/L have been obtained,[7] a more moderate target of 5g/L was chosen to reflect a high but currently achievable product concentration. As was the case in choosing a suitable fermentation volume, it was important that a high MAb concentration be used in order to investigate fully the potential capacity and throughput limitations of packed bed chromatography and also to see if similar limitations exist for the alternative techniques.

All techniques were modelled as batch operations with the required equipment, sized accordingly. Evaluation of techniques under continuous operation was considered to be outside the remit of this particular study. Some of the techniques included in this study may be operated in continuous mode, and in some cases such a property may indeed be considered a significantly attractive feature. Whilst continuous operation is not an explicit attribute within the framework described herein, the framework may still be used to evaluate techniques operated in continuous mode against those operated in batch mode, since it is the impact of operating mode upon process characteristics such as productivity and cost which are of most interest to the process engineer.

Chromatographic techniques (Packed bed, Membrane chromatography and Monoliths) were all modelled in bind and elute mode. For reference purposes, the dynamic binding capacity of the Protein A resin used for the affinity packed bed chromatography technique was assumed to be 30g/L with a resin lifetime of 100 cycles. Resin cost was assumed to be $10,000 per litre.

An example of the distributions used to describe the ratings for attributes within the process performance and the ease of process development groups for three of the techniques considered in this study (ATPE, HPTFF and Protein A Affinity Chromatography) are shown in Table 5.6.

Table 5.6: Example of the Probability Distributions and Values Used to Describe Three Bioseparation Techniques (ATPE, HPTFF and Affinity Chromatography) in Terms of Attributes Within the Process Performance and Ease of Process Development Attribute Groups

		Distributions		
Attribute Group	Attribute	ATPE Rating	HPTFF Rating	Aff. Chrom Rating
Process Performance	Yield	Triangular (Min = 60, Likeliest = 85, Max = 90)	Triangular (49, 66, 82)	Triangular (86, 90, 94)
	Purification Factor	Triangular (1, 3, 9)	Triangular (9, 17, 32)	Triangular (90, 100, 110)
	Resolving Power	Triangular (50, 55, 75)	Triangular (70, 75, 80)	Triangular (80, 90, 100)
	Concentrating Power	3	8	3
	Productivity	Based on Yield and Process Time (9hrs)	Based on Yield and Process Time (11hrs)	Based on Yield and Process Time (9hrs)
	Capacity	Based on Yield and Equip. Vol. (45,000L)	Based on Yield and Equip. Vol. (38,000L)	Based on Yield and Equip. Vol. (22,000L)
Ease of Process Development	Validation	18	25	23
	QC Effort	Triangular (25, 37.5, 50)	Triangular (20, 25, 30)	Triangular (25, 37.5, 50)
	Performance Parameters	Triangular (50, 62.5, 75)	Triangular (75, 80, 85)	Triangular (75, 87.5, 100)
	Scale Down Models	Triangular (80, 90, 100)	Triangular (75, 87.5, 100)	Triangular (75, 82.5, 90)
	Platform Technology	Triangular (80, 90, 100)	Triangular (80, 90, 100)	Triangular (90, 95, 100)
	Generic Processes	Triangular (25, 37.5, 50)	Triangular (0, 12.5, 25)	Triangular (75, 82.5, 90)

5.4 Results and Discussion

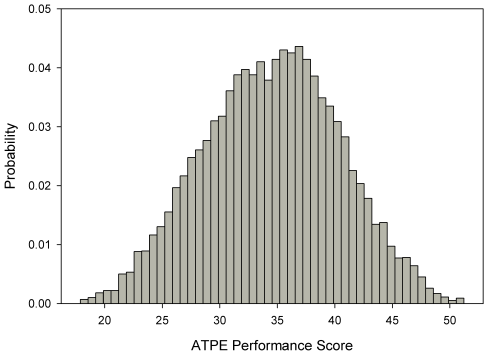
5.4.1 Attribute Group Scores

The MADM analyses were performed in two parts. Firstly each technique received a score distribution reflecting their performance, ease of operation, ease of scale-up, process economics and also ease of process development. An example of the score distributions obtained for Aqueous Two Phase Extraction (ATPE) for each of these attribute groups is shown in Figure 5.5. These charts exemplify the results typically obtained from a non-deterministic analysis with a range of possible scores being returned, as shown along the x-axis of these charts. The height of the bars indicates the number of times each score was obtained over the course of the Monte Carlo Simulations which were run during the MADM analysis. Thus whilst the most likely performance score obtained by ATPE may be approximately 30, it can be seen from Figure 5.5(a) that under certain sets of assumptions or conditions, this score can

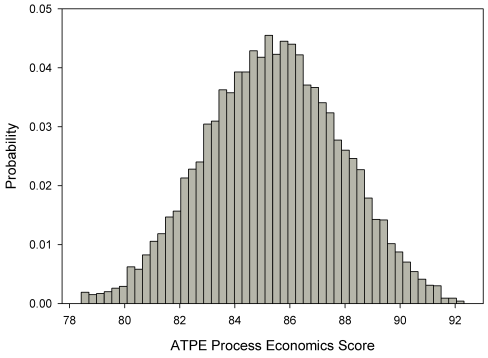
rise to as high as 44.

One way of quickly comparing the scoring of each technique in each of the attribute groups, is to take the average scores obtained and then to plot these onto a scatter chart as shown in Figure 5.6, where techniques are listed along the x-axis, whilst the y-axis shows the score each technique obtained for each of the attribute groups. Figure 5.6 can also be used to see how the different techniques match up against one another in terms of each of the attribute areas. For example it can be seen that in terms of *Process Performance*, Affinity Packed Bed Chromatography scores the highest, whilst Affinity Filtration scores the lowest. The vertical spread of points for a particular technique illustrates the trade-offs which can occur between different process attributes and also the issues which process engineers face when determining the optimal bioseparation technique to utilise in a downstream process. For example, Affinity Filtration and Three Phase Partitioning both show a fairly wide vertical spread of points, implying that whilst these technique score well in certain areas, they also score quite poorly in others. Both obtain high scores for the process economics attribute group because of the moderate capital investment associated with Affinity Filtration and the relatively low consumables costs of Three Phase Partitioning. However the low yields and purification factors achievable using these techniques means that they both suffer from low scores in the Process Performance attribute group.

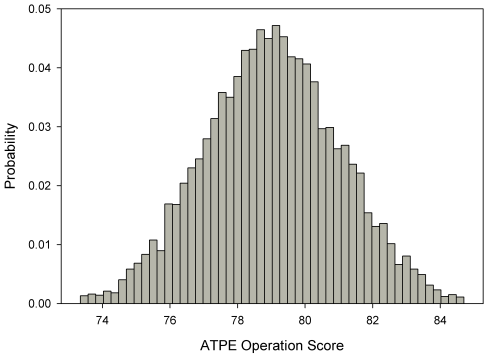
Other techniques such as ATPE also show a certain degree of spread. However from Figure 5.6, it can be seen that for the majority of attribute groups, ATPE actually scores fairly well, with most average scores greater than 50. The majority of points are thus clustered towards the top half of the chart (between 50 and 80). The high Process Economics score is most likely due to the relatively low associated consumables costs (phase recirculation has been assumed). ATPE scores well in terms of scalability and process development since it is essentially a bulk mixing process. As a result scale-up is relatively straightforward. However the average score obtained for ATPE under the process performance attribute group is significant lower than the rest, at approximately 30. This primarily is due to the low purification factors and the moderate yields achieved using this technique.



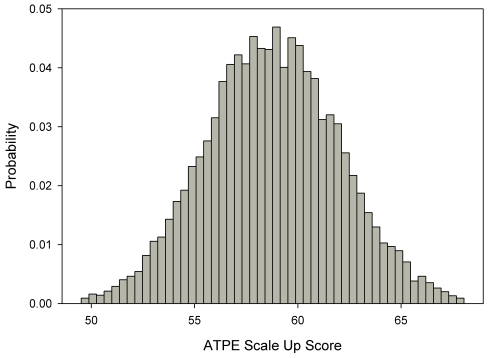
(a) Process Performance



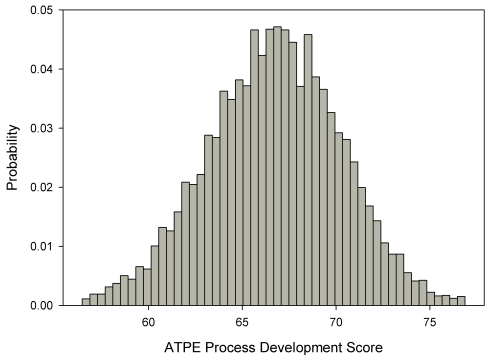
(b) Process Economy



(c) Ease of Operation



(d) Ease of Scale Up



(e) Ease of Process Development

Figure 5.5: Histograms showing the range and distribution of scores Aqueous Two Phase Extraction (ATPE) received for each of the five attribute groups.

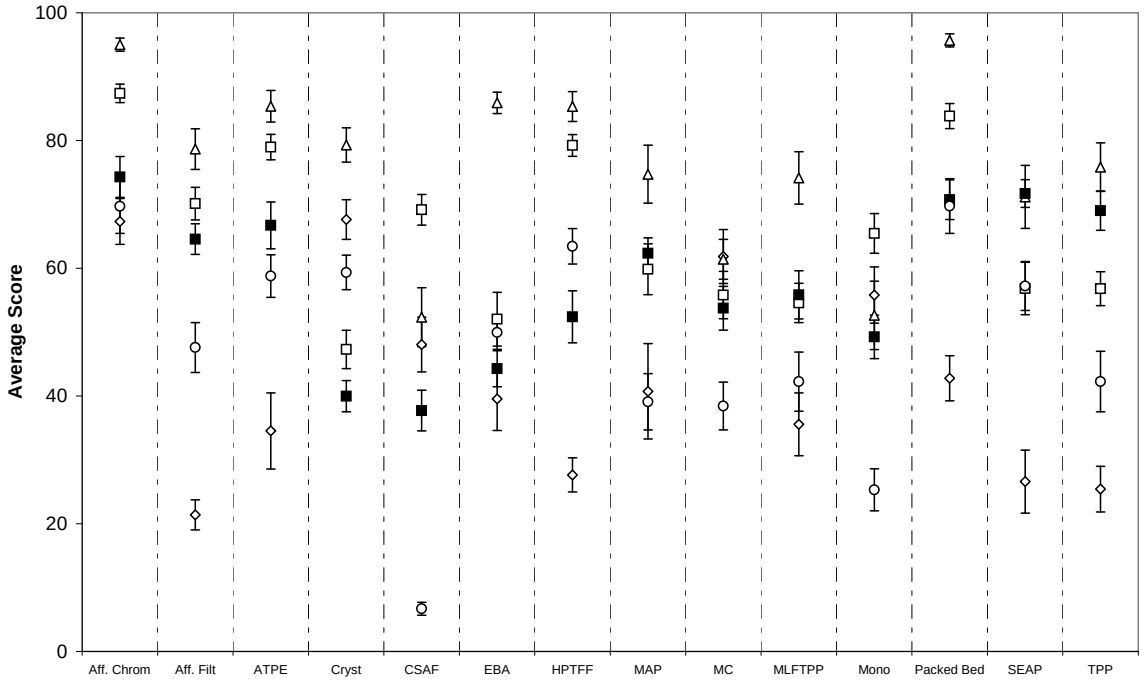


Figure 5.6: Scatter plot showing the average scores each of the bioseparation techniques recieved in terms of performance (◇), ease of operation (□), process economics (△), ease of process development (■) and ease of scale-up (○) attribute groups used in the MADM analysis.
Error bars indicate the standard deviation of scores obtained over the course of the Monte-Carlo simulations

This example presents an interesting case in which, whilst from most perspectives a technique seems to be a fairly attractive alternative, a significant trade-off in one of the process attributes has a profound impact upon the overall attractiveness of the technique. This outcome is of course largely dependent upon the level of importance placed upon the process attribute which has been traded-off, in this case Performance.

The two packed bed chromatography techniques (both affinity and non-specific) score the best in terms of process economics. Alternative technique such as ATPE, TPP and SEAP might be expected to score better in terms of process economics since they do not utilise costly Protein A affinity resins. However the capital costs associated with some of these techniques are significantly higher than packed bed chromatography. For example SEAP requires the utilisation of multiple mixing tanks and phase separators in order to achieve bioseparation, whilst packed bed chromatography only requires a single skid and column. As a caveat, the models used for the majority of alternative techniques were based on data obtained from literature on small scale lab experiments and the conditions may not be optimised for large scale

operation. A good example of this is ATPE, in which the phase compositions used in the small-scale proof-of-principle experiments can result in the requirement for vast quantities of salt and polymer at large scale and lead to ATPE having a relatively high associated raw materials cost and a lower process economics score than packed bed chromatographic techniques.

5.4.2 Overall "Industrial Attractiveness" Scores

From the results of the first part of the MADM analysis, due to the wide range of scores obtained by each technique in each of the attribute groups it is difficult to determine directly which of the alternative techniques is the most "industrially attractive". In order to account for process trade-offs, the scores obtained from the first round of MADM were then used in a second analysis (Figure 5.2; to determine the overall "industrial attractiveness" of the different techniques. The MADM analysis was performed such that the score obtained for each iteration from the first round of MADM was fed directly into, and used in, the same numerical iteration in the second round of MADM. Score distributions reflecting the overall "industrial attractiveness" of each of these bioseparation techniques were thus generated.

As was the case for the individual attribute groups, after the second round of MADM analysis, each technique received a distribution of scores reflecting their "industrial attractiveness" which may be represented in a frequency distribution histogram, similar to that shown in Figures 5.3 and 5.5. In order to perform a quick comparison and ranking, the average score obtained by each technique was plotted onto a bar chart (Figure 5.7). The height of the bars represents the average score obtained by each technique, whilst the error bars reflect the maximum and minimum scores obtained across all the Monte Carlo simulations which were performed.

The techniques may be broadly grouped into three "tiers" based on their average attractiveness scores. The first tier is comprised of the packed bed chromatography techniques, both affinity based and non-specific (e.g. IEX, SEC, HIC). These techniques display significantly higher attractiveness scores than any of the other techniques included in this study (70-80). The second tier is comprised of ATPE, HPTFF

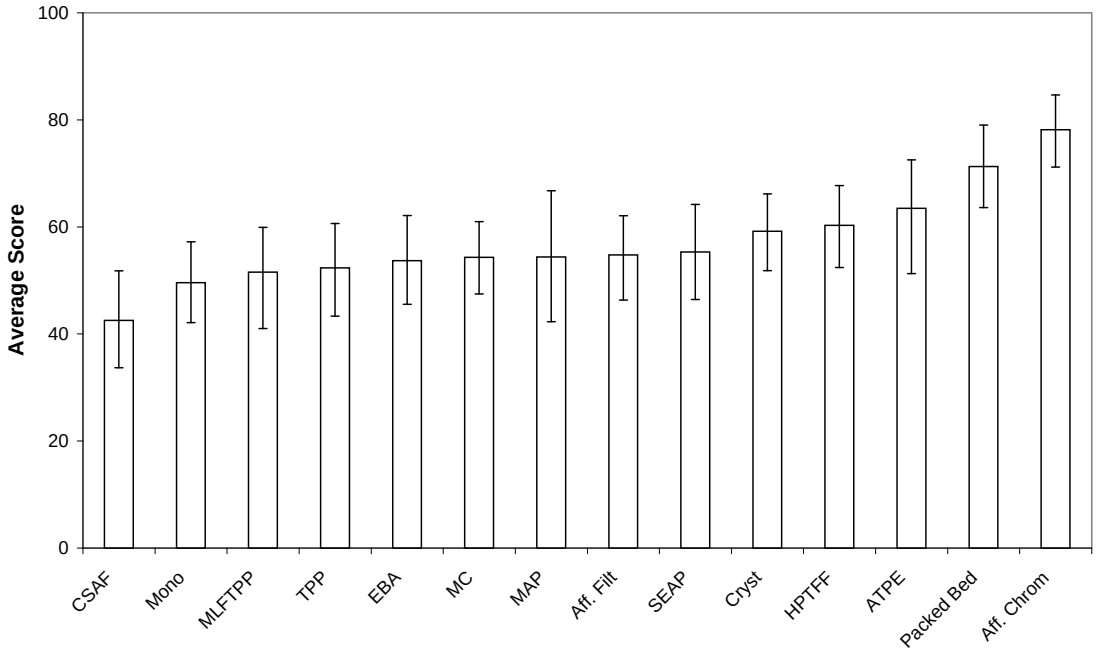


Figure 5.7: Histogram showing the average "industrial attractiveness" of each of the bioseparation techniques and their relative ranking in terms of this metric.
Error bars indicate the maximum and minimum scores recieved over the course of the Monte-Carlo simulations.

and Crystallisation which all displayed average attractiveness scores of between 60 and 65.

The remaining techniques, outside of this "top five" all received highly comparable attractiveness scores ranging between 50-55, with the exception of CSAF which received a significantly lower score (approximately 40), and as such was the least attractive bioseparation technique which was considered in this study.

5.4.3 Accounting for Process Trade-offs

From Figure 5.6, it can be easily seen why packed bed Protein A affinity chromatography was found to be the most attractive bioseparation technique. Based on the use of specific affinity interactions, Protein A chromatography allows for the most selective separation of the all the techniques considered in this study (with the possible exception of Crystallisation)[62] and also typically provides very high yields. Of all the attributes within the performance attribute group, Purification factor and yield were the most highly valued. As a result, the high scores Affinity chromatography received for these two attributes were mostly likely sufficient to outweigh the relative

drawbacks encountered in terms of possible limitations in capacity or throughput and productivity. Overall, affinity chromatography was found to receive, on average, the second highest process performance score. Affinity chromatography is also a well established technique, being almost a pre-requisite for MAb purification, allowing for the development of platform purification processes. As a result, the scale-up of affinity chromatography is relatively well understood, with established rules allowing for straightforward process scale-up. Affinity chromatography also scores well in terms of process economics as described previously.

Based on the results of the bioseparations survey used to gather information for this study, Performance, ease of scale-up and process economics were considered to be the most important attribute groups, and whilst affinity chromatography scored well in all areas, it is in particular the high scores obtained in these three attribute groups which lead to affinity chromatography receiving on average the highest overall attractiveness score.

Non-specific packed bed chromatography was found on average to be the second most attractive technique considered in this study and for reasons similar to those for affinity chromatography. The major differences between these two techniques are that non-specific chromatography resins are in general, significantly cheaper than Protein A resins, with the trade-off being that they are also less selective. From Figure 5.7, it can be seen that the cost saving benefits of using non-selective resins are not perceived sufficient to outweigh the loss in resolving power resulting from the use of non-specific resins.

Combining the results in Figures 5.6 and 5.7, it is possible to determine the reasons behind a technique being particularly attractive or unattractive and in doing so this illustrates the ability of this approach to deal with process trade-offs. For example, ATPE scores well overall in terms of industrial attractiveness, however Figure 5.6 shows that ATPE scores relatively poorly in terms of process performance, due mainly to the limited purification factors which are achievable using this separation technique. Results from the previously mention bioseparations survey showed that of all the attribute groups included in this study, process performance was con-

sidered to be the most important by process engineers. Thus based on the results summarised in Figure 5.6 alone, it would be easy to assume that ATPE would be of only a limited level of attractiveness. However from Figure 5.7 it can be seen that the other positive characteristics of ATPE, such as its scalability, relatively low cost and potential for high capacity operation,[27] are sufficient to outweigh the weaknesses it displays in terms of purifying power. As a result the overall attractiveness of ATPE is relatively high, ranking on average third behind Affinity and non-specific packed bed chromatography. HPTFF is another interesting case, whereby the variation in the scores obtained in each of the individual attribute groups makes it difficult to determine whether or not it is an attractive technique. As was the case with ATPE, HPTFF scores relatively poorly in terms of process performance, mainly due to the long processing times associated with the technique (as a result of the need to use a large number of diavolumes in order to achieve bioseparation) and also the modest yields which can be obtained. However HPTFF receives high scores for the process economics, ease of scale-up and also the ease of operation attribute groups. Membranes are relatively cheap and the operation can essentially be performed in one rig, which explains the high process economics score. Also HPTFF is essentially a form of ultra-filtration utilising a charged membrane. As a result the technique benefits from the fact that it is based on a well understood, well established bioseparation technique and this explains the high scores it obtains in terms of process scale-up and operation. Again, as was the case with ATPE, these benefits are sufficient to outweigh the drawbacks of low yield and productivity In contrast to ATPE and HPTFF, crystallisation scores very well in terms of process performance. This can be mainly attributed to the high purification factors and concentrating power of this technique. However the use of protein crystallisation as a purification technique has been seen to show only limited levels of robustness, with difficulties encountered in developing conditions which provide reproducible results.[62, 109] As a result crystallisation scores poorly in terms of the ease of operation and ease of process development. However due to the high level of importance placed upon process performance, these limitations are not sufficient as to completely dismiss crystallisation as a possible alternative

bioseparation technique. Outside of this top five, the remaining techniques show very comparable levels of industrial attractiveness and which are lower than those of the techniques previously detailed. As indicated in Figure 5.6 it seems that the limitations of these techniques are such that any benefits that they may offer are eclipsed by their inherent limitations. As such, in their current state, the "industrial attractiveness" of these techniques falls significantly short of those of the top five.

5.4.4 Non-deterministic Analysis

Performing the MADM analysis in the way described in the methods and materials and presenting the results in the manner shown in Figure 5.7, allows the overall, most attractive bioseparation techniques to be easily identified based on the average industrial attractiveness scores obtained. Whilst this chart provides insight as to which alternative bioseparation techniques show the most promise, and also how their attractiveness for use in large scale manufacturing processes compares to other alternatives and that of packed bed chromatography operations, further insight may be gained by fully exploiting the non-deterministic capacity of the MADM approach which has been used.

Head to Head Comparisons. The presence of uncertainties within the input data of the MADM analysis means that it is difficult to determine whether a particular technique is unquestionably more attractive than another based purely upon the average attractiveness scores, particularly if these scores are highly comparable. So for example, based on the error bars shown in Figure 5.7, it can be seen that under certain conditions MAP, which ranks amongst the lower half of techniques in terms of average industrial attractiveness, receives a higher industrial attractiveness score than Non-specific packed bed chromatography, the second most attractive bioseparation technique. In another example, SEAP is on average more industrially attractive than Affinity Filtration, however again the error bars on Figure 5.7 indicate that under certain assumptions Affinity Filtration scores higher. A useful way of quantifying and visualising a head to head analysis of these two technique would be to measure the ratio between the attractiveness score obtained by SEAP to that obtained by Affinity

Filtration across the entire range of Monte Carlo simulations performed during the MADM analysis. Iterations in which ratios greater than 1 are obtained would represent those in which SEAP was found to be more attractive than Affinity Filtration, whilst iterations in which ratios lower than 1 are obtained would represent the reverse.

Plotting the results of such an analysis, a frequency distribution histogram may be generated as shown in Figure 8. The range of ratio values obtained is shown across the x-axis whilst the frequency at which each ratio value was obtained during the Monte Carlo Simulations is shown along the y-axis. Blue bars represent iterations in which SEAP was found to be more attractive than Affinity Filtration whilst red bars represent iterations in which the opposite was true. Thus it can be seen that under almost 50% of the conditions tested during the MADM analysis, Affinity Filtration was found to be more industrially attractive than SEAP. This would imply that under a significant number of processing conditions Affinity filtration is actually more attractive than SEAP, and as a result depending upon the circumstances of the process engineer, it may be worthwhile considering Affinity Filtration over SEAP.

Treatment of the results of the MADM analysis in the manner shown in Figure 5.7 provides a quick impression as to the relative attractiveness of the techniques. For example from Figure 5.7, it can be easily seen that CSAF is never more attractive than packed bed chromatography. However when techniques receive comparable average attractiveness scores and/or achieve overlapping score ranges, comparing such techniques in the manner shown in Figure 5.8 allows observations as to the proportion of conditions under which one technique is more industrially attractive than another. Thus not only can it be seen whether one technique is "better" than another but then also how much better it is.

Normalisation Scheme Modification. Similar analyses may be performed for all of the technique included in this study in order to make head to head comparisons between these bioseparation technologies. However in order to make the comparisons fair, a slight amendment must be made to the normalisation scheme described previously in which the normalisation of the ratings for each of the attributes was based upon the range of values observed across all of the techniques (Equation 5.3). A

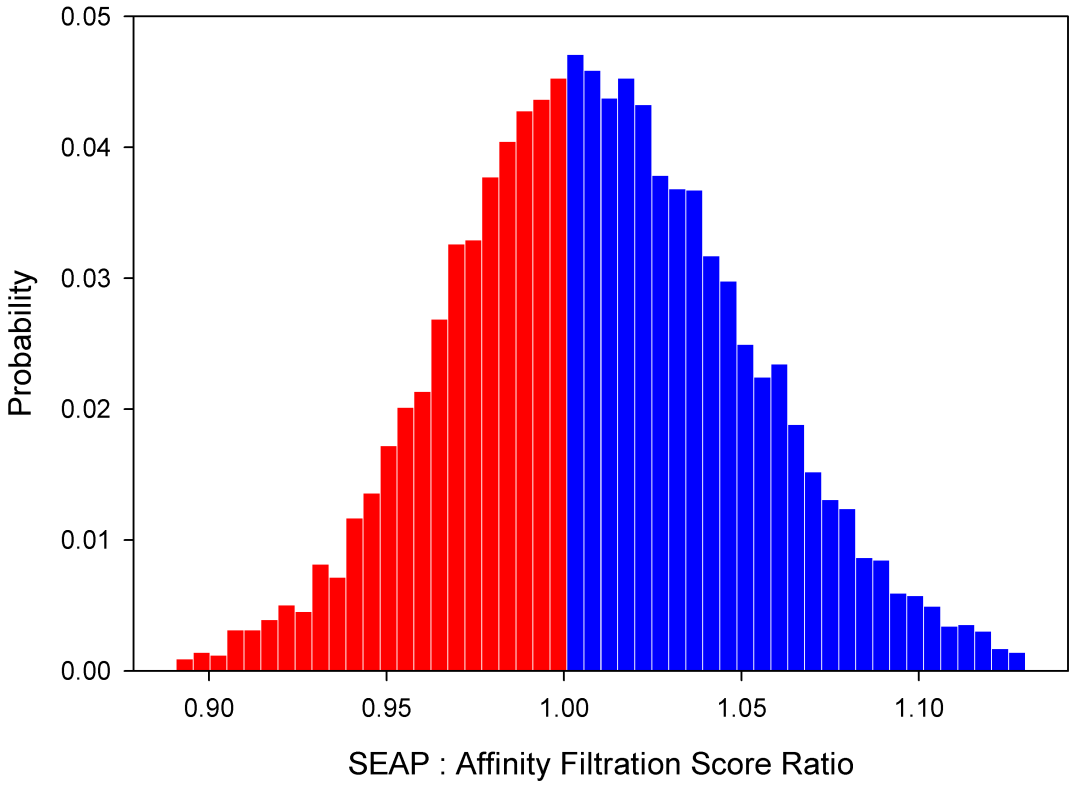


Figure 5.8: Histogram showing the frequency distribution associated with the ratio between the industrial attractiveness score of SEAP to that optained by affinity filtration, over the course of the Monte-Carlo simulations

The x-axis represents the value obtained by dividing the industrial attractiveness score of SEAP by that of affinity filtration. Blue bars represent ratios > 1, red bars represent ratios < 1.

problem arises with such a scheme when a particular operation has an extreme value (x_{ij}) for one of the attribute categories when compared to the rest of the techniques (e.g. a particularly low purification factor or high capital cost etc.). In such cases the normalised ratings (r_{ij}) for all of the techniques in this attribute category will become "polarised". The technique with the extreme rating will obtained a normalised rating of either 1 or 0, with the rest of the techniques scoring at the other end of the spectrum. As a result, differences between techniques may become masked due to the "skewing" of the normalisation range. A wide normalisation range for a particular attribute is not an issue if the values displayed by the different techniques form a distribution across this range. For example, if the worst yield was 25% and the best yield was 95%, this would not be a problem if the yields displayed by the techniques were spread between these two extremes as the significance of the differences in yield between techniques would be accounted for, based on this range. The problem arises when only one technique out of all those included in the study displays a value of 25%, whilst the rest of the techniques display yields of, for example, greater than 75%. Differences between techniques may then be masked by the extension of the normalisation range, caused by what is essentially an "outlier".

In order to perform a more representative head to head analysis of these techniques, in the manner previously described in which the ratio of attractiveness scores between techniques is measured, the extraneous impact of the ratings given to alternatives aside from those being compared must be removed. In order to do this the analysis of the ratio between the attractiveness scores between any two bioseparation techniques was performed using the following normalisation scheme. For attributes in which high values represent better performance, normalisation was performed by simply dividing the value (x_{ij}) by the best value observed across both techniques. For attributes in which lower values represent better performance (e.g. Capital costs, raw materials costs, scale-dependent parameters etc.), normalisation was performed by dividing the best (i.e. lowest) value observed across both techniques by the value for each technique. Thus in both cases, for each attribute, at least one of the techniques received a normalised rating of 1.

Ratio Matrix. The MADM analysis was performed with the altered normalisation scheme and the ratios between the scores for each combination of techniques was recorded. The results of this analysis are summarised in Figure 5.9, which shows a matrix of frequency distribution diagrams. Here techniques are matched up against one another in terms of their overall industrial attractiveness, with the ratios being calculated as the attractiveness score attained by techniques along the rows (A) divided by those along the columns (B). The x-axis on the frequency distribution diagrams in Figure 5.9 therefore represents the values of A/B with the y-axis representing the frequency distribution or probability of a particular ratio being obtained. As was the case in Figure 5.8, red bars represent iterations in which A/B < 1 whilst blue bars represent the iterations in which A/B > 1. This matrix of histograms therefore allows direct comparisons between any two techniques to be made.

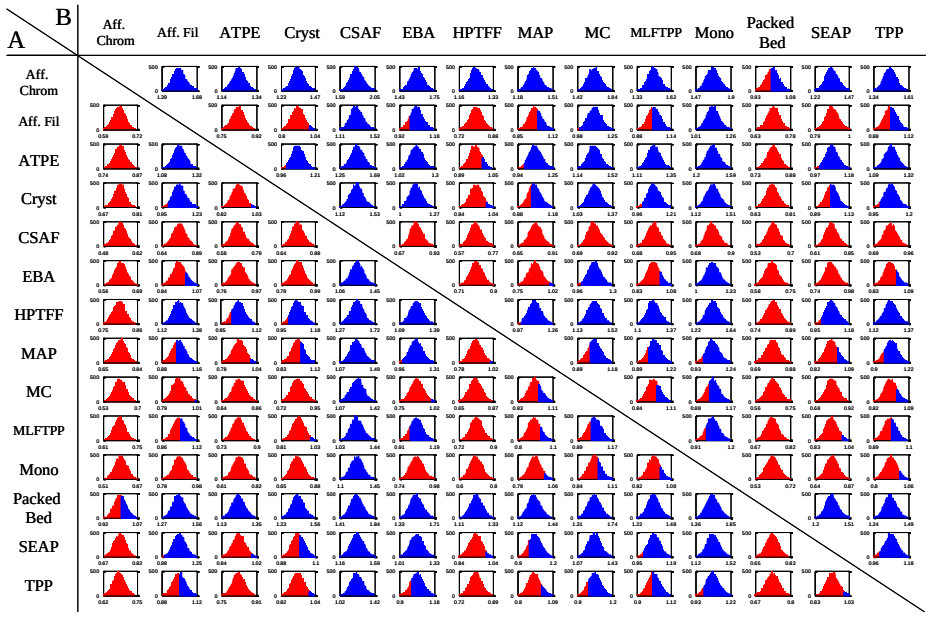


Figure 5.9: Matrix of probability distributions showing the ratio between the attractiveness scores received by different techniques over the course of the Monte-Carlo simulations.
Ratios are calculated as the score of Techniques A divided by that of Techniques B.

Interestingly, some of the results generated through this treatment contrast some-

what with those detailed previously. Figure 5.7 indicated that overall ATPE was more industrially attractive than HPTFF. However the direct comparison of these techniques indicates that under the majority of conditions tested (approximately 90%) HPTFF is actually the more attractive technique (albeit only slightly, as indicated by the relatively narrow range of ratios seen). This implies that ATPE benefited from the aforementioned effects of normalisation range extension by some other technique(s), and represents an example whereby taking the results shown in Figure 5.7 in isolation may be misleading. This however does not negate the relevance of the results shown in Figure 5.7. Performing the MADM analysis using the overall normalisation scheme allows for the significance in the differences between techniques to be accounted for. As a result, ranking the techniques according to the average score obtained in the manner shown in Figure 5.7 may be considered fairer in this regard. The disadvantage of this approach is the possibility of the scores being skewed due to extreme values extending the normalisation range. In performing the head to head analysis, such issues are avoided as techniques have been studied in isolation. The impact of differences between techniques is based solely upon the importance weightings assigned to the attributes and as a result such an approach does not account for the significance of differences when seen from a wider perspective. Both approaches therefore offer their individual advantages and should be used in concert in order to gain the most insight.

5.4.5 Sensitivity and Scenario Analysis

By recording the ratio between the attractiveness scores obtained by different techniques as shown in Figure 5.9 it is possible to observe the proportion of conditions tested during the MADM Monte Carlo simulations under which one particular technique is better than another. From this it can be seen that there are cases in which a particular technique receives a higher attractiveness score than another under all conditions tested (e.g. Crystallisation vs CSAF). It can also be seen that there are instances in which certain conditions lead to one technique receiving a higher attractiveness score, even if the probability of these conditions being met is unlikely (e.g.

ATPE vs HPTFF). By analysing the result of the MADM analysis it is possible to determine the factors which have the greatest impact upon the ratio of the attractiveness scores between two particular techniques, and also to see the conditions which can make a relatively unattractive technique become more attractive.

5.4.6 Head to Head Comparisons of ATPE and HPTFF

Aqueous two phase extraction (ATPE) and high performance tangential flow filtration (HPTFF) were the two top ranking alternative bioseparation techniques in terms of their industrial attractiveness. However neither technique attained higher industrial attractiveness scores than, both affinity and non-specific, packed bed chromatography, with the ratio between the attractiveness score obtained by packed bed chromatography and that of the two alternative bioseparation techniques, being greater than unity across all of the Monte Carlo simulation iterations as can be seen in Figure 5.9. From this it would therefore seem that even the most attractive alternative bioseparation techniques cannot be considered genuine alternatives to packed bed chromatography for the capture and purification of monoclonal antibodies from a cell culture supernatant feed. Based on this, it would seem that in order to make ATPE and HPTFF more attractive than packed bed chromatography, it will most likely be necessary to adjust the actual uncertainty ranges themselves, beyond those which have so far been defined. The question then becomes to which attributes would these adjustments need to be made, and what magnitude of adjustments would be required in order to make ATPE and HPTFF legitimate alternatives to conventional packed bed chromatography. The non-deterministic outputs of the MADM based framework may be used to provide such insight.

As stated, head to head comparisons between both ATPE and HPTFF with affinity packed bed chromatography reveals that with the attribute ratings and importance weightings used in the MADM analysis, none of the iterations in the Monte Carlo simulation resulted in ATPE or HPTFF attaining a higher industrial attractiveness score. Figure 5.10(a) and 5.10(b) shows the distribution of the ratios obtained when dividing the attractiveness score obtained by affinity chromatography to that obtained

by ATPE and HPTFF respectively.

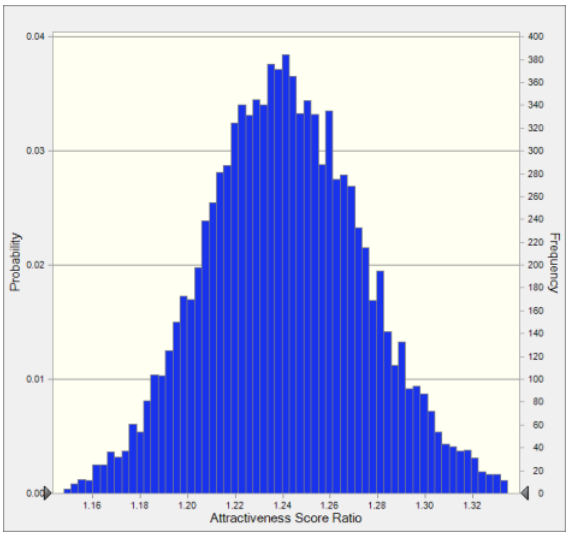
From this it can be seen that the distributions in both cases lie across values greater than 1, indicating that affinity packed bed chromatography outscored the two alternative bioseparation techniques under all conditions tested during the Monte Carlo simulations. With regards to the comparison between Affinity chromatography and ATPE, the average ratio obtained was 1.24, with the full range of ratio values obtained lying between 1.11 and 1.47. In the case of the comparison between affinity chromatography and HPTFF, the average ratio was 1.25 with the full range range lying between 1.13 and 1.36.

The results would therefore indicate that no combination of the currently defined ranges for attribute ratings and importance weightings, results in either HPTFF or ATPE being more attractive that affinity chromatography.

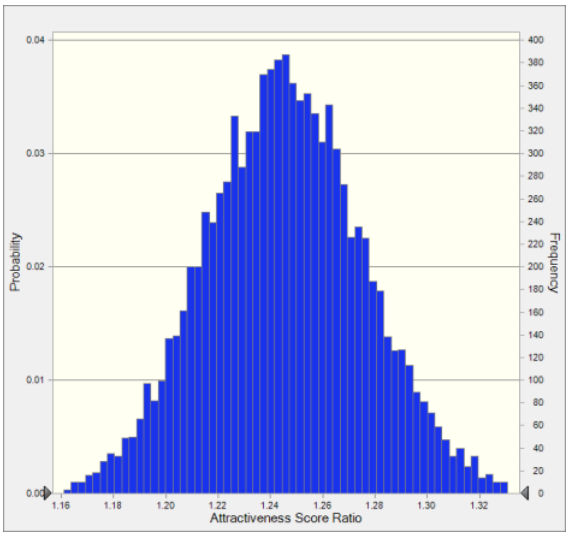
The Tornado diagrams shown in Figure 5.11 show the top ten factors (attribute ratings and importance weightings) which have the greatest impact upon the relative attractiveness scores of affinity chromatography and the two alternative bioseparation techniques, obtained by performing a sensitivity analysis upon the non-deterministic outputs of the MADM based head to head comparison.

The results of the sensitivity analysis presented in Figure 5.11 provides two key insights. Primarily, it serves to indicate the parameters in which greater levels of certainty would result in a more deterministic output from the MADM analysis. Decreasing the uncertainty present in the input variables will inevitably reduce the uncertainty in the output, however the sensitivity analysis helps to determine the precise input parameters which have the greatest influence.

If the ratio between the attractiveness scores of two techniques were to span across values both above and below unity, such information would be useful as it would provide a "target" for development. For example if the yield attribute for one technique, denoted Technique A, were determined to be the major contributor to variance, then decreasing the uncertainty range for this attribute towards higher values would not only narrow the range of attractiveness score ratios obtained , but would also shift the position of the distribution such that the probability of Technique A being more

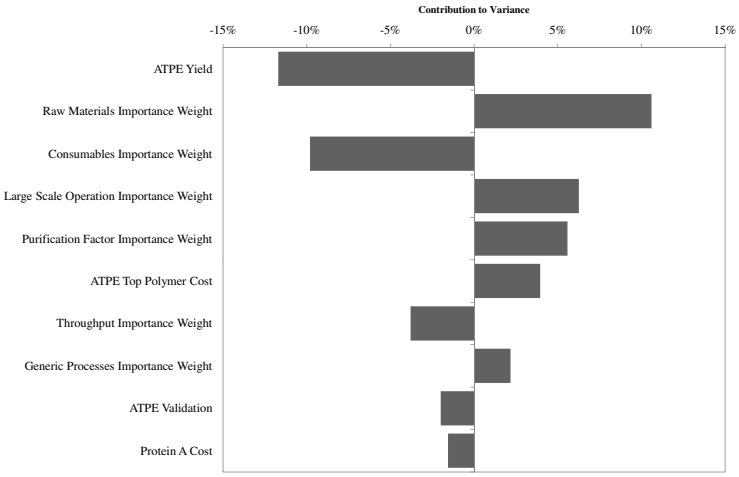


(a) Affinity Chromatography vs ATPE

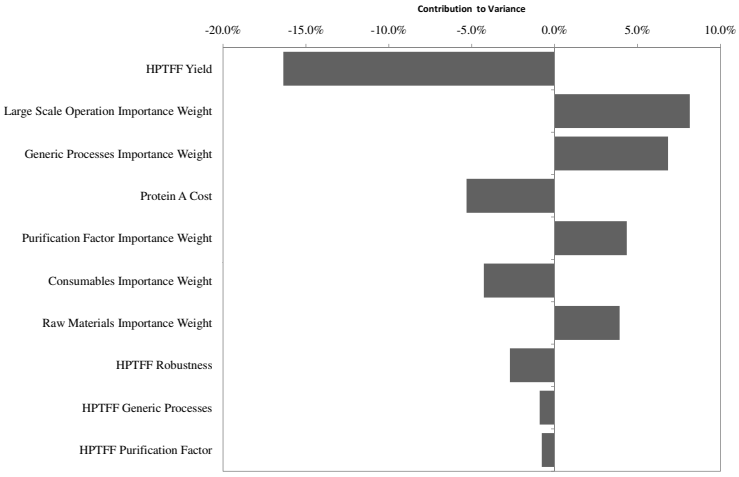


(b) Affinity Chromatography vs HPTFF

Figure 5.10: Frequency distribution of ratios between the attractiveness score of Affinity Chromatography to that of (a) ATPE and (b) HPTFF across MADM Monte Carlo simulations



(a) Affinity Chromatography vs ATPE



(b) Affinity Chromatography vs HPTFF

Figure 5.11: Tornado diagrams showing parameters used in MADM analysis which had the greatest impact on the variance displayed in the ratio values obtained by dividing the attractiveness scores calculated for affinity chromatography by those obtained by (a) ATPE and (b) HPTFF over the course of the Monte Carlo simulations

attractive would also increase. Narrowing the uncertainty range for the yield attribute towards values which benefit Technique A, therefore essentially increases the number of iterations in which it outscores the technique it is being matched up against.

However in this case, the ratio between the attractiveness scores of affinity chromatography and ATPE/ HPTFF is always greater than unity. Thus decreasing the uncertainty present in the input parameters shown in Figures 5.11(a) and 5.11(b) would only serve to indicate exactly how much more attractive affinity packed bed chromatography is compared to either ATPE or HPTFF. For example Figure 5.11(a) shows that the ratio between the attractiveness score of affinity chromatography and ATPE is most sensitive to variability in the value used for the "ATPE Yield" attribute. Moreover, and somewhat expectedly, based on the direction of the bar in Figure 5.11(a), it would seem that the ratio is shifted to lower ratio values with increasing ATPE Yield values. Reducing the uncertainty in this particular input variable to the higher end of the defined range, which in this case is between 60 to 95%, would shift the distribution shown in Figure 5.10(a) to the left. However whilst the range of values spanned by the distribution would become smaller, it will still remain greater than unity, and the probability of ATPE being found to be more attractive than affinity packed bed chromatography will still be effectively zero. Figure 5.12 shows the impact of reducing the uncertainty in the top ten most influential input parameters by 50% towards the ends of the range which are most favourable to the attractiveness score for ATPE. From this it can be seen that by reducing the uncertainty, the ratio distribution has become narrower, and the average ratio between the average scores of affinity chromatography and ATPE has decreased. However the distribution range of ratio values is still greater than unity.

Indeed, taking the top ten input parameters shown in Figures 5.11(a) and 5.11(b), and actually fixing the values at the most desirable ends of the originally defined range, in terms of increasing the attractiveness score of both ATPE and HPTFF, changes the shape and position of these ratio distributions quite dramatically as shown in Figure 5.13. Whereas originally the ratio between the scores of affinity chromatography and ATPE had an average value of 1.24, this has now dropped to a value of 1.06. In

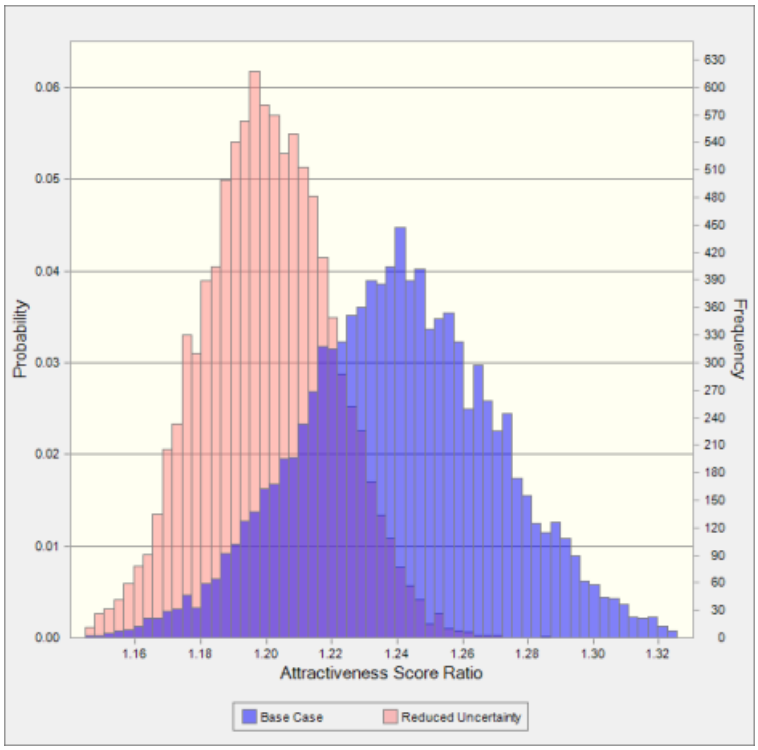


Figure 5.12: Comparison of frequency distributions of the ratio between the attractiveness scores obtained by affinity chromatography to that calculated for ATPE, under base assumptions and under conditions of reduced uncertainty.

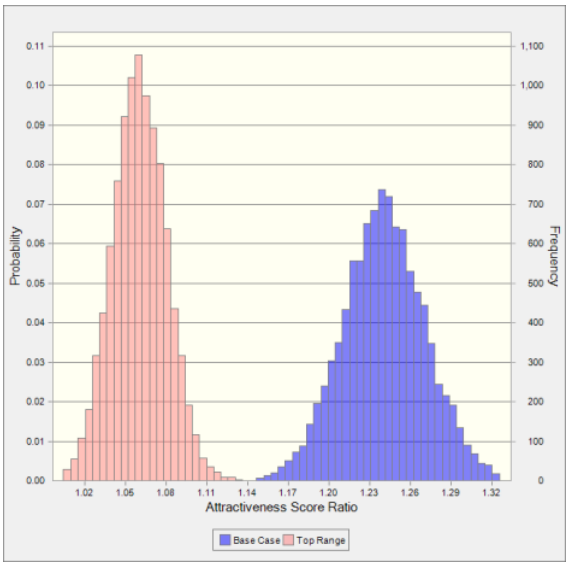
the case of affinity chromatography and HPTFF, the value has dropped from 1.25 to 1.10. However the chances of ATPE and HPTFF being found to be more attractive than affinity packed bed chromatography are effectively zero, with probability values of 0.22% and 0% respectively.

In light of this, the results of the sensitivity analysis serves a second purpose. It is apparent that neither ATPE or HPTFF is more attractive within the constraints imposed by the currently defined ranges of process attributes and importance weightings. Instead, the input parameters will need to be adjusted beyond these ranges, if either ATPE and HPTFF are to have a chance of being found to be more attractive than affinity packed bed chromatography. The use of the sensitivity analysis upon the outputs from the MADM analysis allows the key parameters which must be adjusted to be identified and as a result provide guidance as to the areas in which development work should be concentrated in order to vastly improve the attractiveness of these techniques.

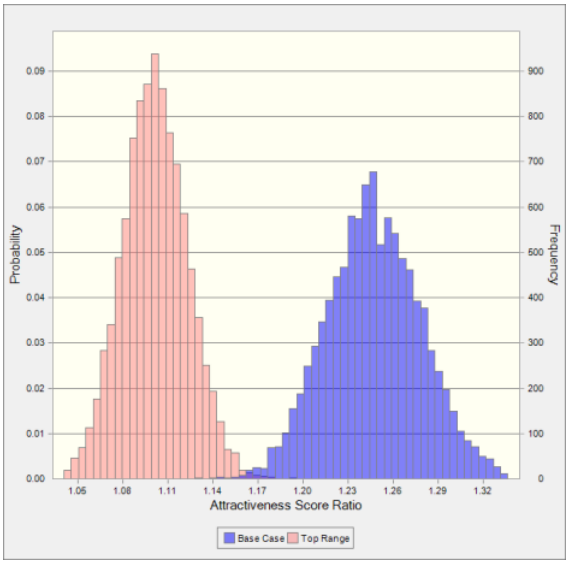
5.4.7 Developing the Industrial Attractiveness of ATPE and HPTFF

Figure 5.11(a) showed that the ratio between the attractiveness score of affinity packed bed chromatography and ATPE is sensitive to variations in the ATPE yield and also the cost of the polymer used to form the two phase system. The maximum yield achievable using ATPE is currently defined as 95% and the lowest polymer price is currently set at 14 £/kg. Based on this, it might be concluded that of all the aspects of the technology which might be developed, the greatest benefit, in term of improving the industrial attractiveness of this technique, would be obtained by concentrating efforts on improving the yields achievable using ATPE to beyond 95%, and also to develop two phase systems utilising polymers with a cost of less than 14 £/kg.

The top ten sensitivity factors may be broken down in two two categories. The first are process attribute ratings. These are process characteristics, and it could be argued are parameters which may be affected by process development effort. The



(a) Affinity Chromatography vs ATPE



(b) Affinity Chromatography vs HPTFF

Figure 5.13: Impact of removing uncertainty from top contributing paramters to variance in the ratio between the attractiveness scores of Affinity Chromatography to that of (a) ATPE and (b) HPTFF

second group of parameters are attribute importance weightings. This group represents the opinions and preferences of the process engineer tasked with evaluating these bioseparation techniques, and as a result can be thought to reflect the processing scenario or context in which the evaluation is being performed. Tables 5.7 and 5.8 show this breakdown for both ATPE and HPTFF.

Table 5.7: Top sensitivity factors for head to head comparisons between affinity packed bed chromatography and ATPE

Rank	Attribute Ratings	Importance Weightings
1	ATPE Yield	Cost of Raw Materials
2	ATPE Polymer Cost	Cost of Consumables
3	ATPE Validation	Precedence of Large Scale Operation
4	ATPE Scale-up	Purification Factor
5	Protein A Resin Cost	Productivity

Table 5.8: Top sensitivity factors for head to head comparisons between affinity packed bed chromatography and HPTFF

Rank	Attribute Ratings	Importance Weightings
1	HPTFF Yield	Precedence of Large Scale Operation
2	Protein A Cost	Generic Process Conditions
3	HPTFF Robustness	Purification Factor
4	HPTFF Generic Conditions	Cost of Consumables
5	HPTFF Purification Factor	Cost of Raw Materials

Importance Weightings

As stated previously, the importance weightings may be thought of as reflecting the opinions of the biochemical engineer and also the processing context for which the bioseparation technique is being evaluated. Fixing the values of these parameters essentially equates to locking the evaluation into a particular context. In this case the

uncertainty in the importance weightings listed in Tables 5.7 and 5.8 was reduced to zero and fixed at the extreme end of the defined ranges originally determined by the survey detailed in Chapter 4. These weightings were fixed at values which resulted in the greatest benefit in terms of the attractiveness of the alternative bioseparation technique, and that had the most detrimental effect upon the attractiveness of affinity chromatography.

Attribute Ratings

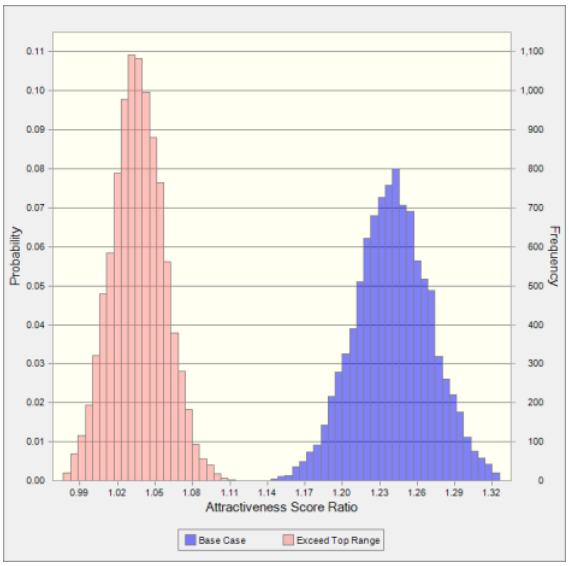
The attribute ratings are based upon the performance of the bioseparation technique, and as a result it could be argued that it is possible these parameters could take values outside of the currently defined ranges at the expense of increased process development effort. In order to determine the effect upon the relative attractiveness of ATPE and HPTFF to affinity chromatography, the values of the top five attributes shown in Tables 5.7 and 5.8 were arbitrarily increased and fixed at values beyond the currently defined uncertainty ranges. For example the yields achievable by both ATPE and HPTFF were both increased from their current levels to 99%. The cost of the phase forming polymers used in ATPE was decreased to \$5 per kg, whilst the cost of Protein A chromatography resin was increased to \$15,000 per litre in both head to head analyses. In the case of HPTFF, the purification factor achievable by the technique was increased to 50-fold, whilst the score for the feasibility of developing generic process conditions was increased to 100.

Figure 5.14 and 5.15 shows the impact upon the frequency distributions for the ratio between the attractiveness score of affinity chromatography to ATPE and HPTFF. Making the previously described adjustments to the attribute ratings can therefore be seen to have increased the relative attractiveness of ATPE and HPTFF, compared to affinity chromatography, that there is now a small probability of both of these alternatives attaining a higher level of attractiveness than the traditional packed bed process. The implication then is that based on the uncertainty present in the input variables, under certain combinations of conditions ATPE and HPTFF could be found to be more attractive than affinity chromatography, when previously the probability

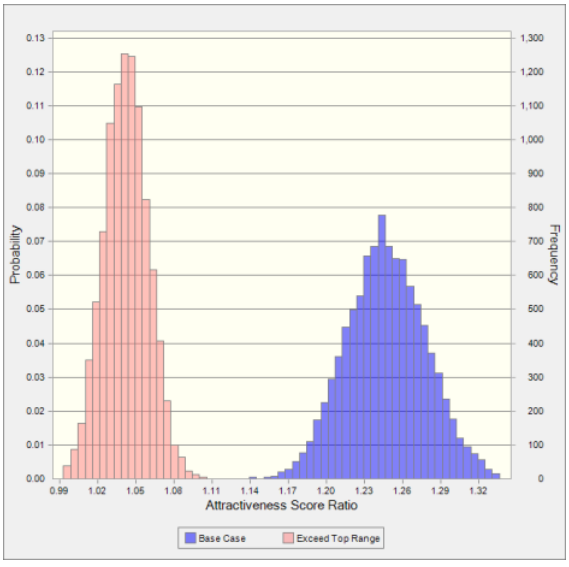
of this occurring was zero. The top five attributes shown in Tables 5.7 and 5.8 therefore not only provide a guide as to which process aspect would most likely cause the alternative techniques to become potentially more attractive than affinity, but the values to which these attributes must attain in order to cause this also provides a set of targets for development.

For example, as described previously, in this revised head to head comparison between affinity chromatography and ATPE, the Protein A cost was increased to \$15,000 per litre, whilst the ATPE polymer cost was decreased to \$5 per kg. It could be argued that affecting an increase in the Protein A price is outside the influence of process development efforts on behalf of ATPE. However an increase in Protein A resin cost could also be easily reflected by a decrease in the associated consumables cost for ATPE. Thus in analysing the results of such an analysis, the most relevant metric for comparison is the ratio between the consumables cost of affinity chromatography to that of ATPE, rather than the absolute values themselves of \$15,000 per litre (or \$75 per cycle assuming a resin lifetime of 100 cycles) and \$5 per kg (which assuming no recycling of polymers is also the cost of PEG per cycle)respectively. Thus in this particular analysis, the small chance of ATPE being found to be more attractive than affinity chromatography is at least partially due to ATPE having a polymer cost which is, on a per cycle basis, only approximately 7% that of Protein A chromatography resin. This value of 7% then provides a target for the development of ATPE systems, as engineers have a guide that if the polymer cost can be reduced to such a relative level then there is a possibility of ATPE becoming more attractive than affinity chromatography.

The results in Figure 5.14 and 5.15 show that the relative attractiveness of ATPE and HPTFF compared to that of affinity chromatography can be increased by reducing the uncertainty present in the top sensitivity factors and also adjusting key attributes to values beyond the currently defined uncertainty ranges. However it can still be seen that even under such conditions, the probability of these alternatives outscoring affinity chromatography are still very small.

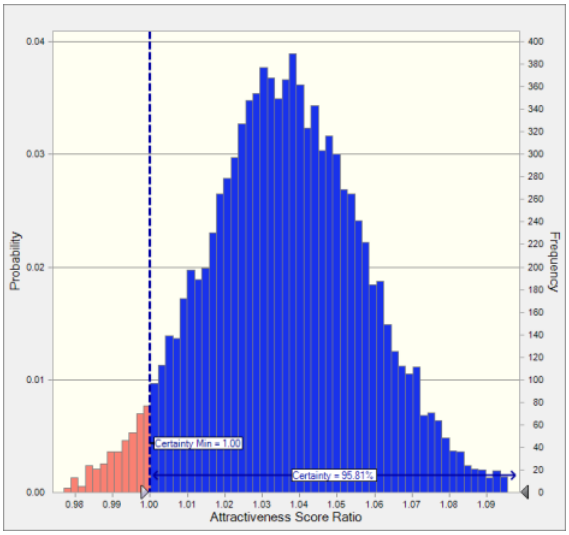


(a) Affinity Chromatography vs ATPE

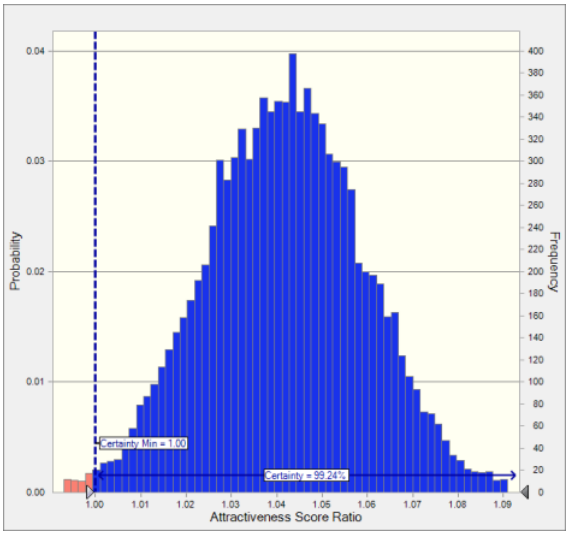


(b) Affinity Chromatography vs HPTFF

Figure 5.14: Impact of adjusting values for top contributing parameters to variance, beyond pre-defined ranges, on the ratio between the attractiveness scores of Affinity Chromatography to that of (a) ATPE and (b) HPTFF



(a) Affinity Chromatography vs ATPE



(b) Affinity Chromatography vs HPTFF

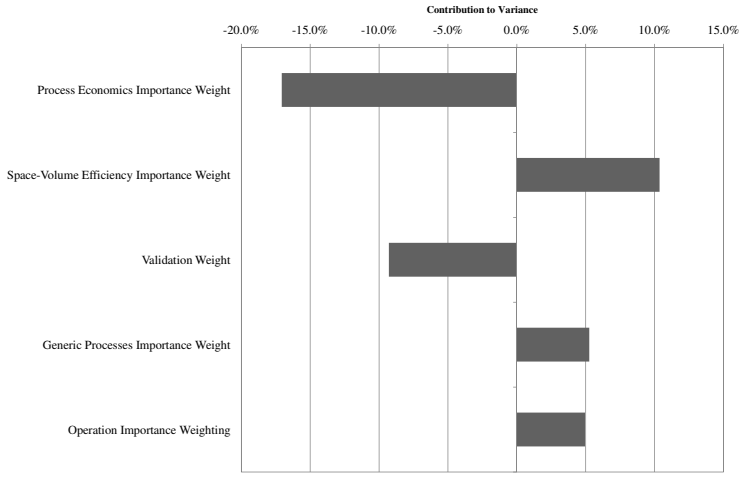
Figure 5.15: Frequency distribution of ratios between the attractiveness score of Affinity Chromatography to that of (a) ATPE and (b) HPTFF across MADM Monte Carlo simulations, under reduced uncertainty and with top variance contributing factors adjusted beyond pre-defined ranges

5.4.8 Further Improving the Industrial Attractiveness of ATPE and HPTFF

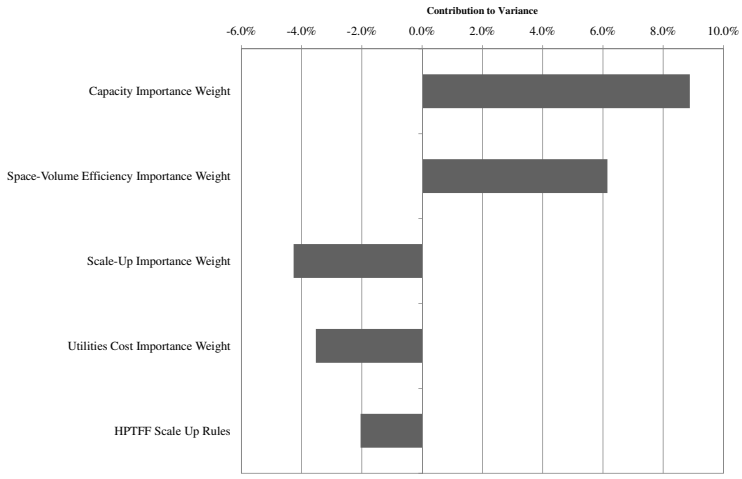
However the fact that there are now iterations in which score ratios of less than unity are being obtained, means that there are some conditions under which ATPE and HPTFF are more attractive than packed bed chromatography, which was not the case in the original analysis. The aim then becomes to determine the parameters which are most responsible for the variance towards these lower values. To address this aim, a second sensitivity analysis was performed under these new conditions of reduced uncertainty and with the top sensitivity parameters from the first sensitivity analysis fixed at values which most benefitted the attractiveness of ATPE and HPTFF. With the top sensitivity parameters identified by the first sensitivity analysis fixed, this second analysis revealed a fresh set of variables, each contributing to the variance seen in the ratio between the attractiveness scores of affinity chromatography, ATPE and HPTFF, the top five of which are shown in the Tornado diagrams in Figure 5.16.

From Figure 5.16 it can be seen that Importance Weighting parameters make up the next five contributors to variance in the head to head comparisons of both ATPE and HPTFF to affinity chromatography, rather than any process attribute ratings. The reason for this could be that for attributes outside of the top five listed in Tables 5.7 and 5.8, the defined value ranges for ATPE and HPTFF are of sufficient difference to that of affinity chromatography, that variations within these ranges do not have a large impact upon the final ratios between the scores of ATPE, HPTFF and affinity packed bed chromatography. Instead it is the level to which these differences are "amplified", through variations in the importance weightings which give rise to the changes in the final score ratio observed.

For example, the importance weighting for the "Availability of Generic Process Conditions" attribute appears in the five contributors to variance shown in Figure 5.16(a). The attribute itself however is not present in the top fifteen contributors to variance made up of the parameters shown in both Figures 5.11(a) and 5.16(a). As described previously, this is most likely due to the magnitude of the difference be-



(a) Affinity Chromatography vs ATPE



(b) Affinity Chromatography vs HPTFF

Figure 5.16: Tornado diagrams showing parameters used in MADM analysis which had the greatest impact on the variance displayed in the ratio values obtained by dividing the attractiveness scores calculated for affinity chromatography by those obtained by (a) ATPE and (b) HPTFF over the course of the Monte Carlo simulations

tween the ratings for this attribute associated with ATPE compared to that of affinity chromatography. Due to the robust nature of Protein A chromatography resins, the attribute rating assigned to this attribute for affinity packed bed chromatography ranged from 80% up to 90%, to reflect the feasibility of adopting generic processing conditions for Protein A processes. Such an approach is not feasible for ATPE processes, due to the process stream specific partitioning behaviour of two phase systems. To reflect this, the attribute ratings for ATPE ranged from only 25% up to 50%. Thus even in iterations in which ATPE scores at the top end of its defined range (i.e. 50%) in this particular attribute, and affinity chromatography scores at the bottom end (i.e. 80%), chromatography still has a sufficiently higher value in terms of this particular attribute rating, that the final impact upon the ratio between the attractiveness scores of ATPE and affinity chromatography is not dramatically altered. This observation brings up an interesting point. One of the primary aims of performing these sensitivity and scenario analyses was to identify key areas in which process development should be focused in order to improve the industrial attractiveness of ATPE and HPTFF relative to affinity packed bed chromatography. The identification of these key areas should not simply be focused on the process attributes revealed by the sensitivity analysis, as these results point to the attributes which are currently "*competitive*" with packed bed chromatography, or at least within close enough proximity that there is an impact upon the relative attractiveness of these techniques. Therefore improvements in these key attributes would result in the most immediate impact upon the ratio between the attractiveness scores of ATPE and HPTFF to that of affinity packed bed chromatography. They would also indicate that since they are already "*competitive*", the level of improvement required is also more modest compared to that which would be required of other attributes. The sensitivity analysis however may also be used to reveal attributes which are currently not yet "*competitive*", however due to the fact that the importance weightings associated with these attributes appear in the sensitivity analysis, would imply that improvements here would also have a sizeable impact upon the relative attractiveness of ATPE and HPTFF, providing further guidance as to areas in which development could be focused.

Taking a similar strategy as was used previously, for the top ten sensitivity factors, the values for these next five major contributors to variance shown in Figure 5.16, for the match ups between affinity chromatography and both ATPE and HPTFF, were fixed at the extreme ends of the currently defined uncertainty ranges and at values

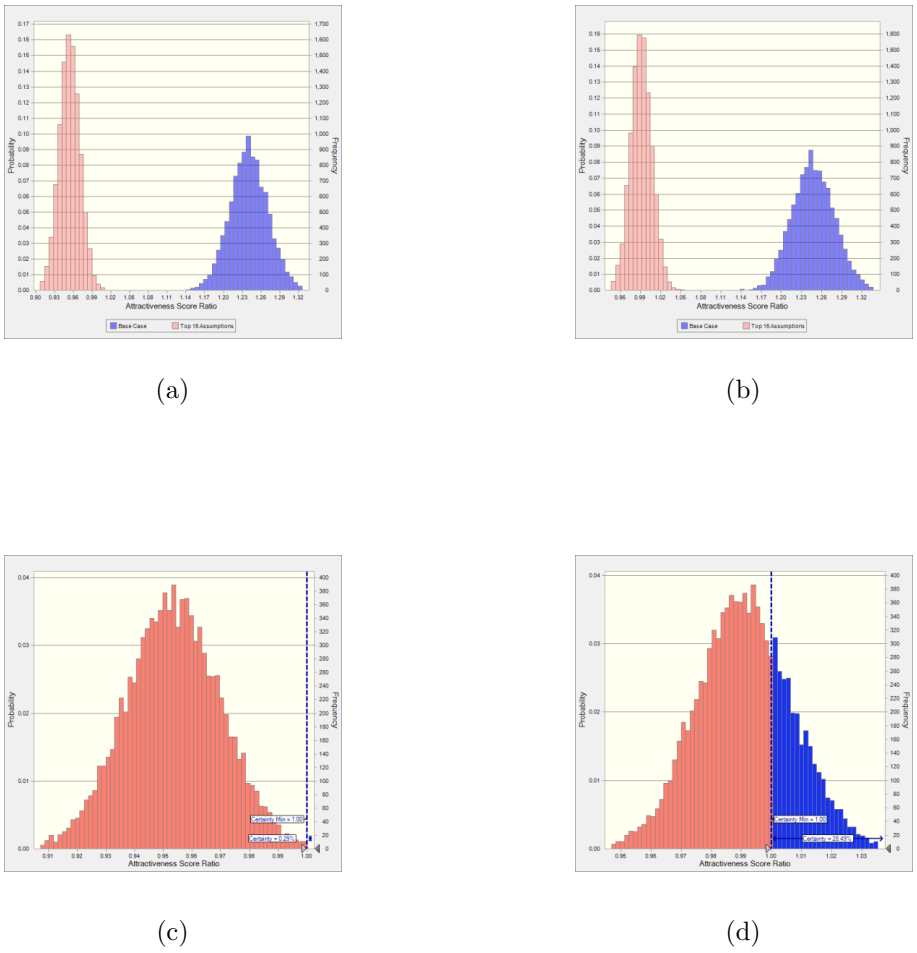


Figure 5.17: Frequency distribution of ratios between the attractiveness score of Affinity Chromatography to that of ATPE and HPTFF across MADM Monte Carlo simulations.
(a) Comparison of frequency distribution for Affinity chromatography vs ATPE under base case condtions, and under conditions in which top 15 parameters have been adjusted to increase the attractiveness of ATPE. (b) Comparison of frequency distribution for Affinity chromatography vs HPTFF under base case condtions, and under conditions in which top 15 parameters have been adjusted to increase the attractiveness of HPTFF. (c) Frequency distribution showing percentage certainty of ATPE attaining a greater attractiveness score than affinity chromatography under "ideal conditions" (d) Frequency distribution showing percentage certainty of HPTFF attaining a greater attractiveness score than affinity chromatography under "ideal conditions"

It can be seen that under these idealised conditions in which the top fifteen contributors to variance have been altered to values which most benefit the attractiveness of the alternative techniques, the probability of ATPE and HPTFF outscoring affinity chromatography has increased dramatically from effectively zero, up to 99% and 70%

respectively. As a result, under these assumptions, the alternative techniques have a greater probability of being found to have higher industrial attractiveness scores than affinity chromatography. The exact values to which the attribute ratings have had to be adjusted to in order to cause this reversal, provide a target for process development. For example ATPE polymer systems must be developed which have only a fractional consumables cost of 0.03% compared to Protein A chromatography, whilst HPTFF processes need to be developed so as to routinely achieve purification factors of approximately 50-fold. The importance weightings used meanwhile serve to indicate the scenarios in which ATPE and HPTFF are most likely to be found to be more attractive than affinity chromatography.

Whilst these results would show that it should be theoretically possible to increase the relative attractiveness of these techniques to beyond that of affinity chromatography with development of a number of key process attributes, it can also be seen that the levels of improvement required are also significant. In order to make ATPE and HPTFF more attractive than affinity chromatography, it has been necessary to adjust many of these key parameters to values well outside of the current defined uncertainty ranges. The presented data however is meant to serve as an illustration of the way in which the framework can be applied to obtain insight as to the ways in which these technique may be improved, rather than a definitive analysis of the future processing potential of ATPE and HPTFF. Such an analysis could be performed using the same methodology presented previously, albeit with constraints placed upon the level of improvement achievable in the parameters identified by the sensitivity analysis. The capability of the MADM framework to allow for rational identification of parameters which are most likely to improve the industrial attractiveness of these alternative techniques however is the key feature which has been shown.

5.4.9 Sensitivity Analysis to Inform Business Related Decisions

To this point, the alternative bioseparation techniques ATPE and HPTFF have been evaluated, and indicators as to areas in which process development should be centred, have been identified. The results of the sensitivity analysis and the use of these results to direct investigations of different scenarios through the utilisation of Monte Carlo simulations, has applications beyond just process development related concerns however. The ability to study the impact of variations in certain parameters upon the overall attractiveness of a bioseparation technique means that it can also potentially be used as a business development tool. For instance a chromatography resin supplier may use such an approach to determine effective marketing strategies for their products.

The usefulness of the tool was illustrated by performing a head to head comparison between affinity and non-specific packed bed chromatography. Further details regarding the results obtained from this study may be found in Appendix A.

5.5 Conclusions

In this study a methodology based on a Multi-Attribute Decision Making (MADM) approach was proposed which can be used to evaluate quantitatively the relative "industrial attractiveness" of bioseparation techniques which may be considered as potential alternatives to packed bed chromatography. A major concern with the adoption of such technologies is the potential for wasting resources on researching and developing techniques which ultimately may not yield significant beneficial returns. The purpose of this study was to develop a tool which could be used to provide a certain degree of guidance as to the techniques which currently show the most promise for use in large scale manufacturing, and thus those on which developmental work should be concentrated. It was intended that any such tool should account for the inevitable trade-offs which will occur between the strengths and limitations of these different techniques. The framework allows a numerical score to be returned reflect-

ing the "attractiveness" of a given technique for use in a large scale biomanufacturing process. The framework was designed such that this quantitative "attractiveness" score reflected the trade-offs between a range of different process characteristics, all of which in some way impact upon the applicability of a particular technique for large scale biomanufacturing. Thus process characteristics such as performance, economics, ease of process development, ease of operation and ease of scale-up were all accounted for. These scores permit the techniques to be ranked in terms of their attractiveness, and thus for techniques which show the most promise to be identified. Based on the results of this study several techniques, such as ATPE, HPTFF and Crystallisation, show a significant degree of potential for large scale operation. However the difference in industrial attractiveness of these techniques compared to that of packed bed chromatographic operations (both non-specific and Affinity), implies that there may still be a long way to go before these techniques may be considered as true alternatives to packed bed chromatography. The ability of this methodology to generate non-deterministic outcomes, means that it can be used for more than simply ranking the techniques in terms of their industrial attractiveness. All of the bioseparation techniques included in this study possess certain strengths as well as drawbacks. In some cases these drawbacks may be difficult to express quantitatively. The usefulness of the approach outlined in this study, is that not only may these drawbacks be dealt with in a quantitative manner, but through appropriate treatment of the non-deterministic outputs, the key strengths and weaknesses of a particular technique can be identified. Such insights may then be used to provide indications as to the areas in which a technique would benefit most from further developmental work, as illustrated by the head to head comparisons between ATPE, HPTFF and packed bed chromatography presented in this chapter.

Based on the results presented, it would seem that the ATPE and HPTFF have a significant amount of ground to make up, if they are to match the industrial attractiveness of affinity packed bed chromatography. Indeed the results would seem to imply that their performance needs to be improved beyond that which has currently been achieved using these techniques. Given the large number of areas in which im-

provements would be required, in order to make these techniques competitive with affinity chromatography, it is unlikely that a single new technological discovery (e.g. inexpensive and easily recyclable polymers for ATPE) or confrontation with a particular process application would result in either ATPE of HPTFF being found to be more attractive than affinity chromatography. This is evidenced by the fact that it was necessary to adjust the values of the top *fifteen* contributors to variance in order to make it more likely that an engineer would find ATPE and HPTFF more attractive than affinity packed bed chromatography. Furthermore, only approximately one third of these top fifteen parameters are actually process attributes, with the remainder being importance weightings. Thus even if the technical aspects of these alternatives could be improved to the levels required, it would still be necessary for the opinions of the engineer to match the required importance weightings for these techniques to become more attractive than affinity packed bed chromatography.

It would therefore seem that improvements in a number of areas is required for these alternative techniques to match the industrial attractiveness of packed bed chromatography. Indeed the predominance of importance weightings in the top contributors to variance would seem to suggest that the defined ranges for certain process attributes are so far apart for the alternative techniques and affinity packed bed chromatography, that variations within these relatively small ranges do not have a large contribution to the variance observed in the final calculated score ratio. Instead it is the amplification of these differences through the importance weightings which have the greatest impact, explaining their presence in the top sensitivity factors. However the presence of these importance weightings in the results of the sensitivity analysis can be used as indicators. Thus whilst for example the "Availability of Generic Process Conditions" attribute does not show up in the sensitivity analysis on the score ratio between ATPE and affinity chromatography, the fact that the importance weighting for this attribute does, would indicate that this is an attribute in which the value range associated with ATPE must be increased to become more comparable to that of affinity chromatography. In this way, the analysis performed and presented in this chapter helps to provide rational guidance as to the areas in which the efforts for

improvement of these alternative bioseparation techniques should be focused.

The evaluative approach outlined in this study therefore can be used not only to provide information regarding the current attractiveness of these bioseparation techniques, but also to suggest possible ways in which this attractiveness can be increased. Given the fact that the industrial applicability of many of these alternative bioseparation techniques currently falls significantly short of that of packed bed chromatography, the mainstay of current downstream processing, the need for further development is clearly evident. The results of the type of analysis outlined in this study should provide insight as to the areas in which development is most needed for each of these techniques. Only by improving the performance of these operations in these key areas can any of these bioseparation technologies be considered as genuine alternatives to packed bed chromatography.

It should be noted that the comparison of the techniques performed in this study was based on applying these for the primary capture of a MAb product. As is almost always the case when dealing with the purification of biopharmaceuticals, the applicability or attractiveness of a technique will depend upon a number of different factors, including the type and characteristics of the target molecule and also the position within the process train in which it is to be used. For example it would be equally feasible to perform the same evaluation for techniques being used as intermediate purification, or polishing steps for a plasmid DNA product, with the attribute ratings and importance weightings changed to reflect the shift in the context of the evaluation. The analysis presented in the study and the conclusions which have been drawn should therefore not be thought of as being a comprehensive assessment but instead an example of the way in which the MADM based methodology may be applied to a specific processing circumstance, and the nature of the insights which may be gained through analysis of the outputs. It is hoped that practitioners may be able to take this framework and tailor the input parameters to suit the particular processing scenarios in which they are most interested.

One potential limitation of the MADM based framework and its outputs which have been described in this chapter is the fact that it assesses these bioseparation

techniques in isolation rather than as part of a whole process sequence. This may in turn mask the true potential of these alternative bioseparation techniques. For example a single alternative may display only modest level of impurity clearance. As a result when evaluated alone, the attractiveness of this technique is low compared to that of techniques such as Protein A chromatography, which owing to the use of affinity interactions, is capable of high purification factors. However in a whole downstream process, the purification factor of a single process step is of secondary importance compared to the purification factor of the process as a whole. Thus the modest purification factors displayed by the alternative technique, may actually be sufficient since the remaining steps in the process are capable of removing the requisite amount of impurities. As a result, whilst one of the aims of analysing the non deterministic outputs of the MADM based framework was to provide targets for process development, which if reached would allow the industrial attractiveness of these alternatives to match that of packed bed chromatography, such aims may not necessarily need to be met, so long as the attractiveness of the overall process is sufficiently high. In order to address this issue ATPE and HPTFF, the most attractive alternative identified by the MADM based framework, were taken forward for further analysis, with experimental studies performed in order to evaluate the performance of these techniques when used as part of a whole downstream purification process. Chapters 6 and 7 describe the results of these studies.

Chapter 6

Integration of Alternative Bioseparations Techniques into a mAb Purification Platform Process - Part I

6.1 Abstract

Experimental studies were undertaken in order to evaluate the performance of ATPE and HPTFF when used as part of a three-step monoclonal antibody purification process. A well characterised three step mAb purification process, utilising Protein A, multi-modal and anion exchange chromatography was taken as a base case. Unit operations within this base case were then replaced with ATPE and/or HPTFF. The performance of these alternative containing process trains were then compared to that of the base case, in order to gain insight as to how these techniques behave when used as part of an entire process train. The results of this study would then help address the concerns over the MADM analysis presented in Chapter 5, which evaluated these alternative techniques in isolation, rather than as part of a whole purification process, thereby potentially underestimating their true industrial attractiveness.

The experimental study itself was split into two sections. In the first section, work

was performed to characterise the performance of an ATPE system developed for the purification of a mAb product, and a HPTFF system utilising a charged membrane. This characterisation work was done firstly in order to gain an understanding of the best ways in which to operate these process, and also to determine the ways in which they could potentially be integrated into a three-step purification process. Whilst the evaluation experiments on HPTFF provided desirable and expected results, the experiments performed using ATPE, revealed significant deviations from the expected behaviour. In light of these deviations, development work was performed in order to understand better the causes of these deviations, and also to try and make the ATPE system work in the desired manner. The insight gained from these experiments was used to inform decisions on how best to integrate ATPE and HPTFF into the three-step mAb purification process.

6.2 Introduction

In Chapter 5, a Multi-Attribute Decision Making (MADM) based framework was used in order to evaluate the relative attractiveness of a number of different alternative bioseparation techniques for use in large scale biomanufacturing processes. Given the high resource requirements associated with process development, the aim of this previous study was to develop a tool which could be used to help identify the most promising alternatives available. This tool allowed bioseparation techniques to be scored in terms of their applicability for use in large scale manufacturing processes, accounting for a wide range of different process characteristics, encompassing attributes such as their performance, associated process economics, scalability, ease of operation and ease of process development. An advantage of adopting a MADM approach in generating this framework was that it allowed the trade-offs between different process attributes to be considered. Thus a technique with a high yield but also high associated operating cost could be compared to a technique with a comparable yield, lower operating cost but protracted process development timelines, in order to determine which was overall the most industrially attractive. The frame-

work was applied to determine the most attractive bioseparation techniques for use in the purification of a mAb product. From this study, Packed bed chromatography, ATPE and HPTFF were identified as being amongst the most industrially attractive techniques evaluated.

The MADM based framework developed, utilised a mixture of both quantitative and qualitative data to describe the performance and characteristics of the techniques evaluated. A statistical summation of all of these different characteristics allowed an overall industrial attractiveness score to be calculated for each technique. One criticism which could be levelled at the evaluation performed in this aforementioned study is that the input data to the developed framework was based entirely from information which could be gathered from available literature rather than any self generated experiment results.

Literature regarding the application of many of these alternative bioseparation techniques to the purification of mAbs is relatively limited. The reason for this is probably two-fold. Firstly the effectiveness of Protein A for the purification of mAbs over the past decade has been such that there has not been a sufficiently large incentive for the investigation of process alternatives. It is only recently that the potential capacity limitations of packed bed chromatography have come into question, giving rise to increased interest in these so-called alternatives. This leads on to the second reason behind the relatively modest amount of literature available on the use of these alternatives for the purification of mAbs. Organisations involved in the purification of mAbs would be understandably reluctant to fully disclose such data due to the possible competitive advantage which such information may confer. What literature there is, either provides details of un-optimised processes, or optimised processes using artificial, or non-representative (for current day standards) feed materials. As a result of these limitations, the performance of some of these techniques with regards specifically to the purification of mAbs had to be extrapolated from what information was available. For example input data regarding the yields achievable by some techniques were based on those observed for target molecules other than mAbs, such as enzymes and other recombinant proteins. Thus an argument which may be levelled

against the findings of this previous study is that the input data did not represent the full potential of many of these alternative bioseparation techniques and that the fully realised industrial attractiveness of these alternative bioseparation techniques for mAb purification has not been evaluated.

A second concern with the findings of this previous study is that the techniques were evaluated in isolation rather than as part of a complete process train. Managing the interactions between the different unit operations of a downstream purification train, ensuring the optimal performance of all steps, is a key element in the process design. By studying the techniques in isolation, it could be argued again that the full potential of many of these alternatives has not been evaluated. For example whilst ATPE alone is not capable of providing the same purification performance alone as Protein A affinity chromatography, it may be the case that an ATPE operation combined with HPTFF and membrane chromatography steps can give the same purification performance as a conventional three column platform process, which would significantly boost the individual industrial attractiveness of these alternatives. The findings of the previous study neglected the impact of such process interactions and integration.

In light of these limitations, the aim of the study detailed in this chapter, was to address these concerns by taking the techniques identified as being the most industrially attractive, namely ATPE and HPTFF, and performing experiments in order to determine firstly how these technique perform in the purification of mAbs and furthermore with feed materials more representative of that which may commonly be obtained from modern cell culture processes. In doing so it was hoped that a more accurate evaluation of process attributes such as yield and purification factor could be obtained. Such parameters, if significantly different from those used in the previous study, could then be used in a second-pass evaluation of these techniques, in order to determine their true suitability for use in the large scale manufacturing of mAbs. Secondly, it was desired that experiments be performed such that observations could be made of the ways in which these alternative processes interact when placed in an actual purification train, thereby allowing these techniques to be evaluated from a

whole bioprocess perspective.

In order to meet these aims, the approach used in the study, was to take an existing, well characterised, Protein A based platform process and use this as a bench-marking base case. ATPE and HPTFF were then used to replace certain unit operations within this base case, generating a number of different purification trains. The performance of these new process trains were then compared to the performance of the original base case in order to determine the effectiveness of incorporating these alternative bioseparation techniques into a platform process, with particular attention being paid to the impacts upon the final product quality, overall processing costs and productivity as well as more qualitative factors such as the ease of process development and operation.

This approach of adapting a platform process to incorporate the alternatives represents a logical starting point, given the extensive characterisation and understanding of these platform processes, but also is a strategy that most likely represents the most ideal scenario from the perspective of a biomanufacturer. The purification of mAbs has been built upon the use of this Protein A platform for the past decade. Therefore the idea of completely retrofitting manufacturing plants with completely novel process trains would clearly be undesirable. In fact the extensive remodelling of plants to incorporate changes in the downstream processing train is one of the drivers for seeking alternatives to packed bed chromatography. The idea of replacing one or two steps within this platform, whilst maintaining the overall structure of the process flowsheet is therefore a slightly more feasible option. From a regulatory perspective it would also be much easier to validate a single change in an established downstream process than a completely novel purification train.

6.2.1 The Base Case

Figure 6.1 shows the typical steps, which a form a Protein A based mAb platform purification process. The base case bench mark process was not made up of all the unit operations shown in Figure 6.1, but instead simply consisted of a Protein A affinity chromatography step for primary capture, followed by a multi-modal ion exchange

step for intermediate purification, followed finally by a anion exchange chromatography step for polishing. This base case had been developed and well characterised previously at GE Healthcare using a number of their commercially available resins. Whilst this process was well characterised and had undergone a certain degree of process development, it could *not* be considered a fully optimised process. The alternative bioseparation techniques were then incorporated into this three-step platform process by replacing one or more of the chromatography columns present in the base case. The alternatives however were not inserted at random points in the process train, but were instead placed at positions in the platform which would theoretically maximise their strengths.

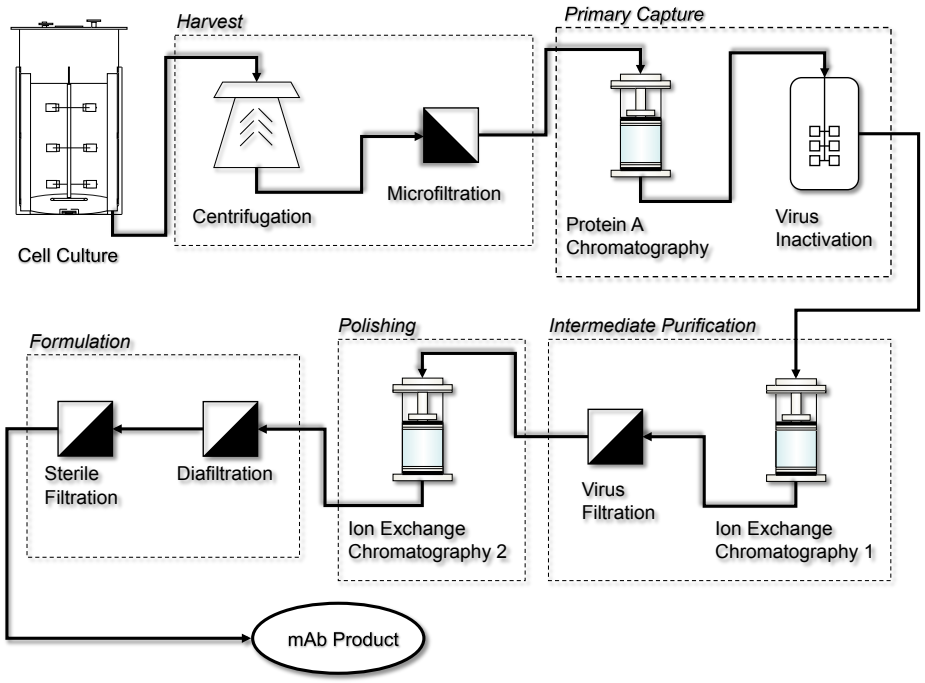


Figure 6.1: Schematic diagram showing typical mAb platform process

6.2.2 Aqueous Two Phase Extraction

ATPE is a bulk separation technique capable of dealing with large fluid volumes and also fairly crude protein mixtures. It has also been shown, unlike packed bed chromatography, to be capable of handling solids containing feeds. These characteristics

make it perfectly suited for use as an early capture step. The modest resolving power afforded by ATPE however, limits its use at any later stages of the purification process train. For these reasons, ATPE was used to replace the Protein A capture step in the three step base case process.

6.2.3 High Performance Tangential Flow Filtration

This membrane based bioseparation technique allows for potentially higher productivities than packed bed chromatography, whilst also allowing for high selectivities and purification factors to be achieved. Indeed data from a past study has suggested that HPTFF may be used to resolve proteins which differ in less than one amino acid.[66] Whilst such a claim must be taken with the caveat that this particular study was performed with a simple two component mixture, HPTFF has been shown to be capable of separating proteins with less than a three-fold difference in size (compared to traditional ultrafiltration processes which are limited to separations of solutes that differ by ten-fold in size), using more representative feed materials.

Unlike ATPE, HPTFF may be of only limited use at early stages of the process train, since crude feed materials may lead to excessive membrane fouling, severely impacting upon process performance. Instead its selectivity and high productivity make it more suited for use either as an intermediate purification or polishing step. Indeed, the inventors of the technology have previously demonstrated the application of HPTFF as a final polishing step. In this process HPTFF was used as part of a three-step process train for the purification of a mAb product. This process train consisted of two ion exchange chromatography processes followed by a final HPTFF step.

In this example, the HPTFF process was shown to be capable of providing a purification factor of greater than 25 fold, resulting in a final product pool with a HCP composition of less than 0.6 parts per million (ppm). This is significantly lower than the highest HCP concentration typically allowable in a mAb product, which is 5 parts per million.[17] As a result it was felt that the full purification capacity of HPTFF was being under utilised by employing it as a final polishing step. Instead,

it this study, the decision was taken to employ the HPTFF step as an intermediate purification step, thereby allowing it to be used not only for purification but also to effect buffer exchange prior to the final ion exchange chromatography polishing process. Based on this logic, the incorporation of ATPE and HPTFF into the base case three-step platform process, in place of packed bed chromatography, results in several different process permutations as illustrated in Figure 6.2.

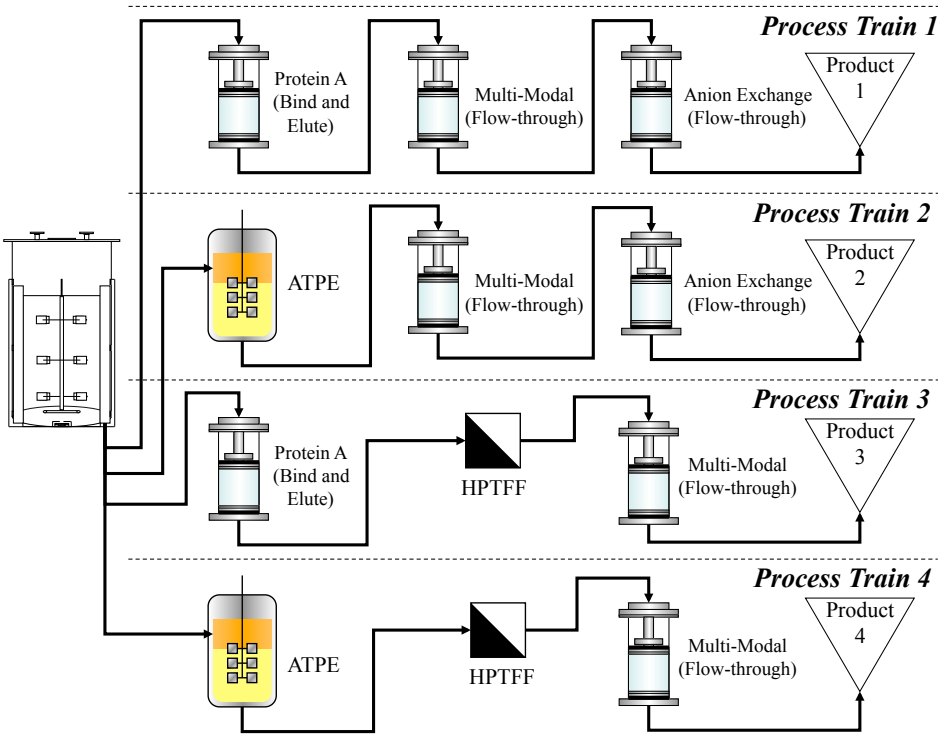


Figure 6.2: Schematic showing possible flow-sheets generated by incorporation of alternative bioseparation techniques into three-step platform process

6.2.4 Process Feed

The processes shown in Figure 6.2 were all challenged with the same feedstock in order to gain a fair comparison between the performances of each process train. The feedstock used was a clarified cell culture supernatant obtained from a culture of CHO cells, expressing mAb at a titre of less than 1g/L. This clarified supernatant was 10 times concentrated in order to generate a feed which had a mAb content, more representative of that which can be achieved by modern cell culture processes. As

described, a concern with packed bed chromatography is the potential productivity limitations which it imposes upon a process when dealing with high titre feed material. By using a feed with a moderately high mAb concentration, it was hoped that any advantages proffered by the use of alternative bioseparation techniques, in terms of throughput could be observed.

Even though ATPE can be used to handle solids containing feeds, since Protein A affinity chromatography requires a clarified feedstock, all of the process flowsheets were challenged with a clarified feed in order to ensure a fair comparison of the process trains. It was felt that this was a good starting point, even though it could be argued that use of ATPE on an unclarified feed could result in a simple primary recovery step.

6.2.5 Process Parameters

Process development is a major issue when dealing with the design of purification processes. This is evidenced by the drive toward the adoption of platform processes beyond simply the purification of mAbs. The use of a platform approach minimises the need for excessive screening of process conditions for process optimisation whilst also making validation of processes much simpler.

A major advantage of Protein A packed bed chromatography, as already discussed, is its high level of process robustness. It is this robustness which allows a platform approach to be used for the purification of mAbs. If an alternative bioseparation technique is to become a feasible option for use in place of Protein A, it must also display at least comparable levels of process robustness. As shown in Figure 6.2, ATPE is being used in place of Protein A for primary capture in Process trains 2 and 4. Instead of developing an ATPE process from scratch and optimising process parameters such as polymer concentration, molecular weight, system pH and ionic strength, the process conditions developed in two previous studies by Andrews et al.[110] and by Azevedo et al.[111] were used. In these previous studies, ATPE processes were developed and optimised for the capture of mAb from clarified cell culture supernatants. The optimised process parameters developed in these two studies (by

Andrews et al. and Azevedo et al.) were then used as the operating conditions for the ATPE processes utilised in Process Trains 2 and 4. It was hoped that by applying a previously optimised process onto a new feed, observations could be made as to the potential robustness of ATPE processes, which in turn would give an impression as to the ease of process development for this particular technique.

With regards to the HPTFF process utilised in Process Trains 3 and 4, the operating parameters used were based on those described by van Reis in his patent concerning the use of charged membranes for the purification of mAbs.[112] In this patent, a potential application for HPTFF is described in which it is used for the final polishing step of a mAb purification train following two packed bed chromatography steps. This scenario is analogous to the processing circumstances into which HPTFF is being placed in Process Trains 3 and 4. As a result the conditions described in this patent were used as the operating conditions for HPTFF in these process trains.

Details of these experimental studies are split over two chapters. Chapter 6 describes the performance of the base case process (Process Train 1 in Figure 6.2) as well as the work performed in order to characterise and evaluate the ATPE and HPTFF processes. Chapter 7 then describes the integration of these alternatives into the platform process, and the overall performance of the resultant process trains.

6.3 Materials and Methods

6.3.1 Feed Material

Chinese Hamster Ovary (CHO) clarified cell culture supernatant was generated in house by GE Healthcare Biosciences R&D (Uppsala, Sweden). The cell line used was obtained from Polymun Scientific (Vienna, Austria). The cell culture supernatant contained a human IgG at a titre of less than 1g/L. This supernatant was concentrated in order to increase the antibody concentration to between 1g/L and 5 g/L.

6.3.2 Chemicals

Polyethylene glycol (PEG), with molecular weights of 1500 and 6000, along with Tris(hydroxymethyl)aminomethane and 3-bromopropyl trimethyl ammonium bromide were obtained in the form of powders from Sigma-Aldrich. Sodium phosphate monobasic (NaH_2PO_4), potassium phosphate dibasic (K_2HPO_4), citric acid monohydrate ($C_6H_8O_7$), tri-sodium citrate ($Na_3C_6H_5O_7$) and sodium hydroxide (NaOH) were also obtained in the form of powders and were purchased, along with isopropanol (C_3H_8O), from Merck Chemicals Ltd (Nottingham, UK). Sodium chloride (NaCl), phosphoric acid (H_3PO_4) and hydrochloric acid (HCl) were all obtained from VWR International Inc.

6.3.3 Base Case Chromatography Process

Protein A affinity capture processes, along with mAb titre analyses were performed using a MabSelect SuRe HiTrap from GE Healthcare Biosciences (Uppsala, Sweden). For the primary capture process, the column was initially equilibrated using 20mM sodium phosphate with 150mM NaCl at pH 7.5. A sufficient volume of cell culture supernatant was then applied to column to give a final load of 28g mAb per litre of resin. The column was then subjected to three separate wash steps, starting with the equilibration buffer, followed by 20mM sodium phosphate with 500mM NaCl and 5% (v/v) isopropanol at pH 7.5, followed by 50mM Tris-HCl with 150mM NaCl also at pH 7.5. Elution was achieved using 60mM sodium citrate at pH 3.4. The eluate from the Protein A capture step was left to incubate at room temperature for 60 mins in order to effect virus inactivation before being titrated to pH 5 using 0.1M NaOH and filtered through a 0.22mm sterile filter. This conditioned Protein A elution fraction was then stored at 4°C. Prior to further processing, the conditioned elution pool was firstly diluted with a 10% (v/v) shot of 250mM sodium phosphate at pH 7. This was then titrated to pH 6.5 with 0.1M NaOH.

The multi-modal ion exchange step used for intermediate purification was performed using a pre-packed Capto adhere HiTrap from GE Healthcare Biosciences

(Uppsala, Sweden). The column was equilibrated using 25mM sodium phosphate at pH 6.5. Feed was then applied onto the column until a load of approximately 179g mAb per litre of resin was reached. The column was then washed with the equilibration buffer with the flow-through during both loading and wash stages, collected at a UV280 absorbance of greater than 100mAU.

The collected fraction from the Capto adhere step under-went buffer exchange using a HiPrep Desalting 26/10 column (GE Healthcare Biosciences, Uppsala, Sweden) prior to being applied to the final CaptoQ polishing step. The HiPrep column was equilibrated using 25mM sodium phosphate pH 7.5. The collected fraction from the Capto adhere step was then applied to the desalting column, and then washed through using the equilibration buffer. Flow through was collected.

A CaptoQ HiTrap column was used to perform the final anion exchange polishing step. The column was equilibrated using 25mM sodium phosphate pH 7.5. A volume of the conditioned feed stream from the previous desalting step was then applied to the column so as to give a final column load of approximately 142 g mAb per litre of resin. The column was then washed with the equilibration buffer. The flow through during loading and wash was collected at a UV280 absorbance of greater than 100 mAU.

A CaptoS HiTrap column (GE Healthcare Biosciences, Uppsala, Sweden) was used to perform cation exchange chromatography as an alternative to the multimodal Capto adhere step used for intermediate purification. The CaptoS column was equilibrated using 20mM sodium citrate with 12mM NaCl at pH 5.3. The feed for the CaptoS column was the conditioned elution pool from the MabSelect SuRe Protein A primary capture step (prior to the addition of the 250mM sodium phosphate shot and titration to pH 7). Feed was applied to the column to give a final load of approximately 100 g mAb per litre of resin. The column was then subjected to two subsequent wash steps following feed loading. 20mM sodium citrate with 150mM NaCl at pH 3.5 was used to perform the first wash step, and 40mM sodium phosphate at pH 6.5 was used to perform the second. A single column volume of equilibration buffer was passed through the column between the two wash buffers. Elution of the mAb was achieved

using 20mM sodium citrate and 200mM sodium chloride at pH 5.3. This elution pool underwent buffer exchange to 25mM sodium phosphate at pH 8.0 using a HiPrep Desalting 26/10 column (GE Healthcare Biosciences, Uppsala, Sweden) as was the case with the Capto adhere product pool, before being applied to the final CaptoQ polishing step. For the CaptoQ step following CaptoS, slightly different buffers were used than that for the CaptoQ step following Capto adhere. 25mM sodium phosphate at pH 8.0 was used for the equilibration and wash steps, with the flow-through once again collected at a UV280 absorbance of greater than 100 mAU.

An AKTA explorer controlled using Unicorn 5.11, (all from GE Healthcare Biosciences, Uppsala, Sweden), was used to perform all chromatography experiments.

6.3.4 Aqueous Two Phase Extraction

Forward Extraction

Two different PEG phosphate ATPE systems were investigated. The first system utilised PEG 1500, with a system composition of 15% (w/w) PEG 1500, 14% (w/w) Phosphate, and 12% (w/w) NaCl. The second system utilised PEG 6000 with a system composition of 12% (w/w) PEG 6000, 10% (w/w) Phosphate and 15% (w/w) NaCl. ATPE forward extraction systems were generated by adding appropriate amounts of PEG, K_2HPO_4, NaH_2PO_4 and NaCl powders directly to the cell culture supernatant feed so as to give the desired system compositions. Systems were formed in 50mL Falcon tubes (BD Biosciences), with system mixing accomplished by placing the falcon tubes onto a rocking platform shaker (custom manufactured at GE Healthcare). Powders were added sequentially with NaCl added first, followed by PEG and finally the K_2HPO_4 and NaH_2PO_4. The monobasic and dibasic phosphates were added so as to give the desired system phosphate mass percentage whilst the pH of the system was controlled by altering the ratio between the mass of monobasic and dibasic salt added. Sufficient time was allowed between the additions of powders so as to ensure complete dissolution of the previously added component before the introduction of the next. Powders were usually completely dissolved after approximately 10 minutes.

Following addition and dissolution of all powders, systems were mixed for a further 60 minutes before being left to settle under gravity for 30 minutes in order to effect phase separation. Systems were then centrifuged at 3000 rpm for 30 minutes, using a Eppendorf 5810R (Eppendorf, Hamburg, Germany) in order to ensure complete phase separation. The top and bottom phases were then carefully separated and the volumes determined. Samples were taken for analysis.

ATPE Back Extraction

Back extraction was performed by taking the top phase from the forward extraction system and adding a back extraction buffer in order to generate a new two phase system. Several back extraction buffers were utilised including a phosphate buffer, made using K_2HPO_4 and NaH_2PO_4, added to give phosphate concentrations of 21-12.6% (w/w) and in a ratio as to give the desired pH, and a citrate back extraction buffer, made using trisodium citrate, with concentrations ranging from 30 - 27% (w/w) and titrated to the desired pH with HCl. Back extraction systems were formed in 50mL falcon tubes, with back extraction buffer added to the top phase, recovered from the forward extraction, in volume ratios ranging from (top phase : bottom phase) 1:2 to 1:1.2. Back extraction systems were mixed in the same manner as the forward extraction systems, using the rocking platform, for approximately 10 minutes. Back extraction systems were then allowed to settle under gravity for 15 minutes, before being centrifuged at 3000 rpm for 10 minutes to ensure complete phase separation

Top and bottom phases were then separated and their volumes determined. Samples were also taken for analysis.

6.3.5 High Performance Tangential Flow Filtration

HPTFF System

The HPTFF system was set-up using a custom combination of an AKTAcrossflow system (GE Healthcare Biosciences, Uppsala, Sweden) and a P-901 pump (GE Healthcare Biosciences, Uppsala, Sweden). The AKTAcrossflow system was set-up with a 350mL

feed reservoir and a small inner diameter tubing kit, used in order to minimise the hold-up volume of the system. The AKTAcrossflow system was used to provide the tangential flow across the charged membrane surface whilst the P-901 pump was used to provide the co-current flow on the filtrate side of the membrane. This co-current flow would be used to balance the transmembrane pressure across the membrane. The AKTAcrossflow was controlled using a PC connected to the system, with Unicorn 5.11 control software installed on it.

In order to achieve the desired operating conditions, a manual pressure controlled valve was added to the retentate recycle loop. Figure 6.3 is a schematic diagram of the HPTFF System. This manual pressure valve was essentially formed of a small length of rubber tubing with an adjustable tubing clamp attached to it. Tightening of the clamp reduced the diameter of the rubber tubing thereby allowing the pressure in the retentate line prior to the valve to be increased.

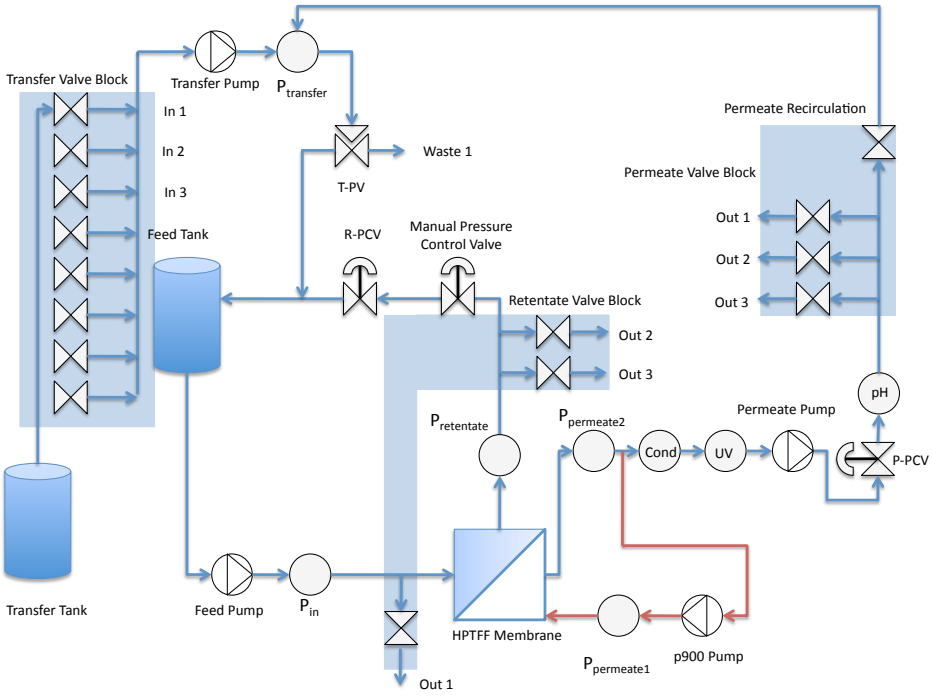


Figure 6.3: Schematic showing set-up of HPTFF system using AKTAcrossflow and P-901Pump. AKTAcrossflow system components are shown in blue whilst red lines represent the co-current flow loop.

Testing of Uncharged Membrane

The sieving properties of the uncharged membrane were tested by challenging it with a protein mixture containing the polyclonal IgG Gammanorm (Octapharma Australia Pty. Ltd. Australia) and bovine serum albumin (BSA) (USB Corporation, Belgium), and determining the resultant concentration of both components in the feed and permeate. HPTFF experiments were performed in total recycle mode with both retentate and permeate returned to the feed tank. Experiments were carried out using the HPTFF system described previously. Feed was delivered to the membrane at a flowrate of 26.9 mL/min (323 LMH). The flux through the membrane was set at the desired level and controlled using the internal feedback controls of the AKTAcross-flow system. The retentate pressure was adjusted to a value of 0.69 bar (10 psi), whilst the co-current flow rate was set in order to achieve constant transmembrane pressure across the membrane. Specifically the co-current flowrate was adjusted such that the difference between the feed pressure (P_{in}) and the bottom permeate pressure ($P_{permeate\ 1}$) was the same as that between the retentate pressure ($P_{retentate}$) at the top permeate pressure ($P_{permeate\ 2}$). The system was left to equilibrate for 15 minutes, before samples of both the permeate and the feed were taken for analysis. The filtration experiments were performed at three different filtrate fluxes of 50, 100 and 150 LMH, whilst maintaining the feed flowrate at 323LMH. Experiments were also performed using two different feeds materials. The first feed consisted of the IgG and BSA dissolved in a 10mM Na-Acetate pH 4.0 buffer, whilst the second feed was made up of IgG and BSA dissolved in 150mM Na-Phosphate pH 7.0 buffer. In both cases, the feed concentration of IgG and BSA was 5g/L and 2g/L respectively.

Adding Charge to Membrane

Charge was added to the membrane using the methods described in the patent by van Reis.[112] Specifically the methods described in Example 1 and elaborated on in Example 6 of this patent. In summary, a reactant solution consisting of 2M (3-bromopropyl) trimethyl ammonium bromide dissolved in 0.1M NaOH was flushed

across and through the membrane. Charging of the membrane was performed using the AKTAcrossflow system. The reactant solution was delivered to the membrane at a feed flowrate of 26.9mL/min (323 LMH). The flux through the membrane was set to 100 LMH and controlled using the AKTAcrossflow feedback control systems. The system was operated in total recycle mode with both retentate and permeate returned to the feed reservoir. Circulation of the reactant solution around the system was continued for 16 hours before the membrane was rinsed with MilliQ water and the charging reaction quenched by flushing the membrane with a 1% (v/v) Acetic acid/ 0.12M Phosphoric acid solution. Co-current flow was not used during the charging reaction.

Testing of Charged Membrane

Characterisation of the charged membrane was performed in the same manner as was done for the uncharged membrane, using the same conditions and operating procedures. As with the uncharged membrane, the charged membrane was challenged with two different feeds. The first consisted of Gammanorm and BSA dissolved in a 10mM Na-Acetate pH 4.0 buffer. The second feed was IgG and BSA suspended in a 25mM Na-Phosphate, 500mM NaCl pH 7.0 buffer. Experiments were performed at filtrate fluxes of 50, 100 and 150LMH.

Normalised Water Flux

Measurements of the normalised water permeability of the membrane were performed prior to use and then after each experiment, in order to monitor the condition of the membrane and to ensure that significant deviations in membrane performance could be accounted for. The normalised water flux was determined using the Unicorn 5.11 (GE Healthcare, Uppsala Sweden) pre-production method wizard.

HPTFF Process

The operating conditions utilised when using HPTFF as part of a whole process train, were based upon the parameters detailed by van Reis in the patent for the use of

charge membranes.[112] Briefly, product pools from the previous purification step were conditioned in order to bring the pH to the required level as well as to reduce the ionic strength and conductivity, thereby maximising the impact of membrane charge upon protein separation. The HPTFF process itself was broken down into two discreet stages. In the first stage the feed was concentrated in order to reduce the process volume to a manageable level. Following concentration, the process stream was then subjected to sequential diafiltration using buffers at pH 4.5, pH 5.5 and pH 6.5. 10 Diavolumes of each buffer were used, with the diafiltration performed at constant retentate volume. All stages of the HPTFF process were performed with a fixed feed flowrate of 323LMH and a membrane flux of 50LMH. The retentate pressure was adjusted to 0.69 bar, whilst the co-current flow rate was adjusted in order to provide equal transmembrane pressures at the inlet and outlet of the membrane cartridge as described previously for the membrane evaluation experiments.

6.3.6 Analytics

A Superdex 200 5/150 GL Tricorn column (GE Healthcare Biosciences, Uppsala, Sweden) was used to perform size exclusion chromatography analysis of experimental samples, in order to determine aggregate content and sample purity. 20mM sodium phosphate with 150mM NaCl at pH 7.4 was used as the running buffer.

A MabSelect SuRe Hi-Trap (GE Healthcare Biosciences, Uppsala, Sweden) was used to perform analytical protein A affinity chromatography of crude experimental samples (i.e. samples prior to Protein A purification) in order to determine mAb concentration and content. 20mM sodium phosphate with 150mM NaCl at pH 7.4 was used for equilibration and washing. Elution was performed using 100mM phosphoric acid at pH 3. A standard curve was generated based on integrated peak areas, using diluted samples of a Polymun IgG standard with a known concentration.

Quantitation of host cell protein (HCP) composition of samples was achieved with a Gyrolab Bioaffy 200 CD microlaboratory using the Bioaffy 200v1 SIA HCP application (Gyros, Uppsala Sweden). Antibodies used in the assay were goat anti-Chinese Hamster Ovary Cell Proteins (IgG at 0.1mg/ml) (Cygnus Technologies, Southport,

NC, USA).

For the membrane evaluation experiments, analysis of IgG and BSA composition of both the feed and the permeate were performed using a Mono Q 5/50 GL anion exchange column. Equilibration was performed using 25mM Na-Phosphate buffer with a pH of 7.2. A gradient elution was used with a 25mM Na-Phosphate, 500mM NaCl pH 7.2 solution as the elution buffer.

Analytical chromatography was performed using an AKTA purifier, controlled using Unicorn 5.11 (GE Healthcare Biosciences, Uppsala, Sweden).

6.4 Results and Discussion

6.4.1 Base Case Process

As described previously, the base case process was a three-step purification train consisting of three different chromatographic operations. The first unit operation was a Protein A affinity chromatography step (using Mabselect SuRe resin) used to capture the mAb from the crude cell culture supernatant feed. This was followed by a multi-modal ion exchange step (using Capto adhere resin) run in flow-through mode for intermediate purification of the mAb. The final polishing step was accomplished using an anion exchange column (packed with CaptoQ), also run in a flow through mode. The performance of each unit operation and the performance of the process train as a whole is briefly summarised.

MabSelect SuRe Protein A Chromatography

HiTrap columns packed with MabSelect SuRe, were used for primary capture. The feed for this step was a concentrated clarified CHO cell culture supernatant, with a mAb concentration of 4.6g/L and a HCP content of 74,750ng/mL or 16250 parts per million (ppm). Feed was loaded onto the Protein A column to a concentration of 28mg mAb per mL of resin and mAb was eluted from the column in approximately 1.8 column volumes (CVs) or 9.16mL. The elution pool from the Protein A column was left to incubate for 60 minutes in order to effect virus inactivation. It was observed

that the elution pool contained some precipitate, spread throughout the body of the bulk fluid. After virus inactivation, the elution pool was titrated with 3mL of 0.1M NaOH to bring the product pool pH to a value of 5.0. Titration of the elution pool seemed to cause further precipitation. The elution pool was centrifuged at 3000rpm for 15 minutes in an attempt to remove the precipitate. However whilst some precipitate was collected in the pellet at the base of the test tube, following centrifugation, the elution pool still had a slightly turbid appearance. The supernatant was decanted and passed through a 0.22mm sterile filter in order to achieve complete clarification. The concentration of mAb in this partially conditioned elution pool was determined spectrophotometrically to be 7.9g/L and the HCP content was determined to be 456ppm, giving the Protein A step a purification factor (PF) of approximately 36 fold. The yield in this elution pool was calculated to be approximately 69% which was deemed to be exceptionally low for a Protein A chromatography process, which can typically be expected to display yields greater than 95%. Initially it was thought that the yield loss was due to the precipitation which had occurred in the elution pool. However analysis of the precipitate showed that it contained very little protein, and was assumed to be made up mainly of lipids, with only trace amounts of mAb, HCP and leached Protein A. Analysis of flow through and wash fractions also showed very little mAb content. It was therefore proposed that the low yield from the Protein A capture step was potentially due to an overestimation of the mAb concentration in the feed caused by some cell culture supernatant media components. Several Protein A chromatography cycles were performed, with the eluates conditioned and pooled, in order to generate sufficient material for subsequent chromatography steps, with similar results obtained across all cycles. 30mL of this semi-conditioned elution pool from the MabSelect SuRe Protein A cycles was taken and sterile filtered. A 10% (v/v) shot of 250mM Na-Phosphate at pH 7.0 was then added to the product pool. The sample pH was then adjusted to 6.5 with 0.1M NaOH. No further precipitation occurred as a result of this additional conditioning. The aggregate content of the product pool at this point in the process was determined to be approximately 0.3%. The fully condition product pool was then loaded onto a Capto adhere 1mL HiTrap

column.

Capto adhere Chromatography

Capto adhere is a multi-modal chromatography resin which can be used to clear a number of impurities from the process stream, including DNA, host cell proteins, leached protein A, mAb dimmers and larger aggregates. In the base case process, the Capto adhere step was operated in a flow through mode, with impurities binding to the column during loading. Feed was loaded onto the Capto adhere column to a concentration of 179mg mAb per mL resin. This is a relatively high concentration, however the multi-modal nature of the Capto adhere resin means that it will always have a certain capacity for mAb binding. Obviously operating in a flow through mode, means that any binding of mAb is undesirable. As a result, having a relatively high load, the yield losses through binding of mAb monomer to the Capto adhere can be minimised. From approximately 28mL of feed to the Capto adhere column, 31.79mL of product containing flow through was collected. The mAb content of the product pool was determined spectrophotometrically to be 5.3mg/mL, and the HCP content was determined to be 169ppm, giving the Capto adhere step a purification factor of approximately 3 fold. The aggregate content of the Capto adhere flow through fraction was determined to be approximately 0.1%. The product pool from the Capto adhere then underwent a buffer exchange step. Whilst at a processing scale, this buffer exchange would most likely be accomplished using a diafiltration operation, in the lab scale experiments performed for this study a HiPrep Desalting column was used. The purpose of the buffer exchange step is to adjust the pH of the product stream (from pH 6.5 to pH 7.5) without increasing its ionic strength and conductivity. Such a pH adjustment may be accomplished by titration of the product pool with NaOH. However this would inevitably increase the ionic strength of the product stream which would in turn hinder binding of components to the following Capto Q anion exchange column. The desalting column is essentially a gel filtration column with a very low exclusion limit, so that only small molecules such as salts may penetrate the pores of the resin particles and be retained, whilst larger molecules, such are proteins are

excluded and pass straight through the column. The differences in accessible volume (and therefore retention time) between the proteins and the salt molecules in the load sample allows for effective separation of the two. The desalting buffer exchange step is, in theory, a non-selective step in terms of protein purification. However in this case an approximately 20-fold reduction in the HCP concentration was observed across the desalting step. The product pool from the Capto adhere step was found to have a HCP concentration of approximately 164 ppm, whilst the product pool from the desalting step (and the load for the subsequent Capto Q step) was found to have a HCP composition of only 8 ppm. It is unlikely that such a high level of HCP clearance could have been achieved over a simple desalting column.

Based on the information which is available, a possible explanation for the high purification factor observed across the desalting step, is that there has been an error in the HCP assay for one of the in-process samples. In this case, it is most likely that the HCP concentration in the Capto adhere product has been overestimated. Capto adhere is a multi-modal anion exchanger and should be capable of providing high purification factors. The 3-fold reduction in HCP which was observed may therefore be considered fairly modest. It is likely that the HCP concentration of the Capto adhere product is closer to that observed in the Capto Q load, and that the HCP clearance observed over the desalting step is actually attributable to the Capto adhere step.

Capto Q Chromatography

Capto Q is an anion exchange resin which is used as a final polishing step in the three column mAb purification platform, to remove final trace impurities in the process stream. Following the desalting step, the concentration of mAb in the feed was determined spectrophotometrically to be approximately 4.4g/L, with a HCP concentration of only 8 ppm. The Capto Q step was able to reduce this HCP concentration further to just over 2 ppm, giving the step a purification factor of approximately 3.4. The yield from the Capto Q step was very close to 100%, which may be expected since the column was operated at a pH well below the pI of the target mAb. No aggregates

were detected in the flowthrough from the Capto Q step. Figure 6.4 shows the purity of the product pool as measured by SEC. The monomer has a retention volume of approximately 2mL. From this it can be seen that the aggregate levels throughout the process are very low.

Table 6.1: Summary of results from base case process

Step	Initial		Final		Yield	PF
	mAb Conc. (g/L)	HCP (ppm)	mAb Conc. (g/L)	HCP (ppm)		
Mabselect SuRe Protein A	4.60	16,250	7.90	456	69%	35.66
Capto adhere	6.29	457	5.30	164	96%	2.79
Capto Q	4.39	8	4.29	2	100%	3.44

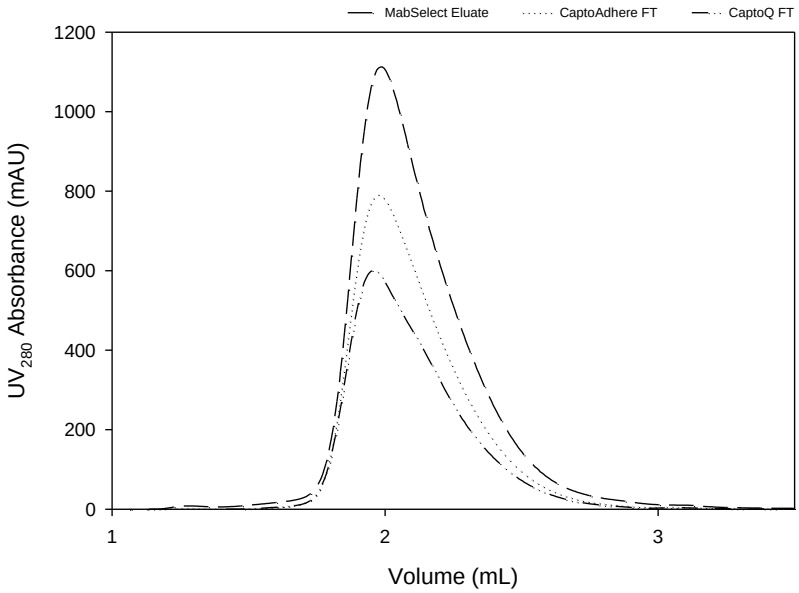


Figure 6.4: Product purity by SEC over the course of the base case process

Overall the three step base case process was able to achieve an overall process yield of approximately 67% and a purification factor of almost 7000 fold. The HCP and

aggregate content of the final product pool were within typical acceptance criteria for a mAb therapeutic product

6.5 ATPE Forward Extraction Development

Before incorporating the ATPE process into the three step mAb purification platform, in place of Protein A chromatography, experiments were performed in order to characterise the exact performance of the ATPE system with the feed material used in this study. An ATPE process itself is split into two discreet stages; forward extraction and back extraction. Phase forming components are initially added to the feed in order to form the forward extraction ATPE system. During this forward extraction the aim is to partition the target molecule, in this case mAb, into the top polymer rich phase. The top phase from the forward extraction is then recovered and mixed with a new salt rich phase to form a second ATPE system. The aim of this second back extraction ATPE system is to recover the mAb from the top polymer rich phase, by causing it to partition into the bottom salt rich phase. The bottom phase may then be recovered and passed on for further processing.

6.5.1 ATPE Forward Extraction

The major difference between the forward and back extraction systems are the relative concentrations of the phase forming components. With the ATPE systems used in this study, the phase forming components are PEG (with a molecular weight of either 1500 or 6000), phosphate salts and NaCl. In PEG - Phosphate ATPE systems, in the absence of NaCl and at a relatively neutral pH (6-7), mAbs have been shown in past studies to preferentially partition to the bottom salt rich phase.[110, 111] This is due to the electrostatic potential difference between the top and bottom phases created by the uneven partitioning of phosphate ions. The high concentration of negatively charged phosphate ions in the bottom phase creates an electrostatic attraction to mAb molecules which will typically have a positive charge at neutral pH values, causing them to move into the bottom phase. The partitioning behaviour of mAbs may be

reversed by introducing NaCl into the system. The addition of NaCl causes two effects. Firstly NaCl partitions evenly between top and bottom phases and as such will increase the ionic strength of the already phosphate rich bottom phase. Increasing the ionic strength of the bottom phase will serve to reduce protein solubility, and as a result the partitioning of mAb to the bottom phase will cease to be thermodynamically favoured as a result of the addition of NaCl to the ATPE system. The presence of negatively charged chloride ions in the top phase also serves to provide an electrostatic attraction to positively charged mAb molecules. The combination of reduced protein solubility in the bottom phase and increased electrostatic interactions in the top phase serve to facilitate mAb partitioning to the polymer rich top phase. The manipulation of these forces forms the basis for the forward extraction ATPE systems, which utilise relatively high concentrations of both phosphate and NaCl in order to encourage partitioning of mAb to the top phase. During back extraction the top phase from the forward extraction is mixed with a fresh salt phase, thereby forming a new two phase system. The bottom salt rich phase of this new two phase system will have a lower ionic strength than in the forward extraction and the overall NaCl concentration of the system will be lower. As a result the partitioning of mAbs to the bottom phase once again becomes favourable and the product can therefore be recovered in the relatively salt rich phase and passed on for further processing.

6.5.2 Impact of Order of Powder Addition

The forward extraction step of the ATPE process was accomplished by adding NaCl, PEG 1500 and phosphate salt powders to the clarified cell culture supernatant feed, such that the final system composition was 15% (w/w) PEG, 14% (w/w) Phosphate and 12% (w/w) NaCl. In order to ascertain whether the order in which these powders were added to the supernatant had any discernible impact upon the performance of the forward extraction step, several different ATPE forward extraction systems were set-up with the powders added to each system in a different order. The results are summarised in Table 6.2.

The order in which the powders were added to the supernatant did not seem to

Table 6.2: Impact of order of powder addition upon ATPE system characteristics

Order of Powder Addition	Partition Coefficient	Phase Ratio (V_t/V_b)	mAb Yield Top Phase	mAb Yield Bottom Phase
PEG - PO4 - NaCl	389.09	0.9	164%	0.6%
PEG - NaCl - PO4	375.65	1.0	154%	0.5%
PO4 - PEG - NaCl	450.84	1.0	163%	0.5%
PO4 - NaCl - PEG	744.75	1.0	163%	0.4%
NaCl - PEG - PO4	848.70	1.0	153%	0.5%
NaCl - PO4 - PEG	713.48	1.0	157%	0.5%

have a significant impact upon the partitioning of feed components. Regardless of the order in which the salts were added, mAb preferentially partitioned to the top phase with partition coefficients (K) greater than 100. mAb yields in the top phase also showed a high level of consistency across all experiments. Whilst the order of powder addition did not greatly affect these system properties, it did have subtle impacts upon the physical behaviour of the system.

In all the systems tested, an interfacial precipitate formed at the interface between the top and bottom phase of the forward extraction system. The point during the forward extraction at which this precipitate initially forms was dependent upon the order of powder addition. Adding either PEG or Phosphate salts first to the cell culture supernatant caused immediate precipitation. If the addition of PEG was followed by the addition of NaCl, then this precipitate could then be made to resolubilise. However this was not the case if the addition of Phosphate salts was followed by the addition of NaCl. Addition of either PEG following Phosphate, or Phosphate following PEG, caused the already present precipitate to become thicker, and also seemed to cause a significant increase in system viscosity, to the point where the system could not be effectively mixed using a vortex mixer. Adding NaCl first did not cause the formation of any precipitation. Addition of PEG following NaCl did also not result in any precipitation. However the addition of Phosphate salts following NaCl did cause a precipitate to form.

As stated the order of additions did not have a discernible impact upon the par-

titioning behaviour of process feed components, which would be a primary concern. However the formation of precipitates may have processing implications, particularly when considering the use of ATPE at large scales. Precipitates can entrap process fluid, which in the case of an ATPE system would be portions of top and bottom phase. At a small scale, this is not a significant issue, since high speed, batch centrifugation of the test tubes, housing the ATPE systems, allows highly efficient separation of all three phases (top phase, interfacial precipitate and bottom phase). Thus the amount of fluid entrapped in the precipitate would be relatively small.

However at a large scale, the use of batch centrifuges is not practical and phase separation would need to be accomplished most likely using continuous disc stack centrifuges. Here complete phase separation and solids dewatering would not be possible, at which point the amount of product containing top phase entrapped in the precipitate could possibly become an issue, potentially leading to unacceptable yield losses. For this reason, it might be beneficial, from a large scale processing perspective, to add the powders in an order so as to delay the precipitate formation to as late a stage of the forward extraction process as possible, in order to minimise the level of fluid entrapment. In which case, the optimal order of powder addition would be NaCl, followed by PEG and finally followed by the Phosphate salts.

6.5.3 Interfacial Precipitate and Mass Balance

As stated previously the aqueous two phase forward extraction process resulted in the formation of an interfacial precipitate which collected between the top and bottom phase following centrifugation. This precipitate proved to be difficult to resolubilise in a range of various buffers including PBS, sodium citrate and phosphoric acid. As a result it was not possible to determine its exact composition. In this situation the main concern is that this precipitate may contain a certain amount of mAb, representing thus an irrecoverable yield loss. However it was not possible to close the mass balance on mAb in the top and bottom phases of the forward extraction system, due to either an underestimation of the mAb concentration in the feed, or an overestimation of the mAb concentration in the top phase, resulting in mAb yields of approximately 150%

in the top phase. The high mAb yield is unlikely to be due to an underestimation of the feed concentration (since if anything the 4.6g/L concentration is most likely to be an overestimation based on the Protein A yields from the base case). Instead, it was assumed that the high recovery was due to an overestimation of the amount of mAb in the top phase.

The mAb concentration of the top phase was determined using analytical Protein A chromatography in conjunction with a standard curve generated using a sample of Polymun IgG with a known concentration. The IgG concentration of top phase samples were determined from the integrated peak areas of the analytical Protein A chromatogram. The overestimation of mAb concentrations in the top phase may be a result of some interference between the ATPE system components and the assay method. For instance the presence of PEG in the load sample may be causing increased levels of non-specific binding of process related impurities, leading to increased elution peak areas. This would then cause an overestimation of the mAb concentration.

Due to the inability to determine the precise mAb concentration of the top phase, it was not possible to determine the mAb content of the interfacial precipitate at this stage.

6.5.4 Differences in Forward Extraction System Performance

In the original study by Andrews et al.[110] in which this ATPE system was developed, the formation of an interfacial precipitate during the forward extraction is briefly alluded to. It is stated that the interfacial precipitate was found to contain some IgG (approximately <10%) and that this was successfully resolubilised and recovered during the back extraction. Whilst no clear details are given with regards to the exact amount of precipitate present in the forward extraction system, the fact that it could be resolubilised indicates a divergence in system behaviour between that described by Andrews et al., and that observed in this study. Whilst the system composition, with regards to PEG, NaCl and Phosphate has been maintained, the characteristics of the feed material show some substantial differences. Firstly the mAb concentration

is significantly higher in this study. The cell culture supernatant used in the previous study contained an IgG concentration of 1.5g/L, whilst that used in this study had a titre of approximately 4.6g/L. Also the concentration of host cell proteins (HCP) is different. Whilst the feed used by Andrews et al., had a HCP concentration of approximately 6 million parts per million (ppm) mAb, the feed used in this study only had a HCP concentration of approximately 16,000 ppm. Thus the feed to the ATPE systems used in this study was significantly purer than that used previously. There may also be other differences between the feeds, for example the concentration of lipids which can also directly influence the level of precipitate formation.

6.6 ATPE Back Extraction Development

The back extraction system is described somewhat briefly by Andrews et al, and there is a significant level of ambiguity with regards to the system definition in terms of phosphate and NaCl composition. In this study, the back extraction system developed by Andrews et al., was interpreted as being accomplished by adding a 21% (w/w) phosphate stock solution, formed with appropriate amounts of mono and dibasic phosphate salts so as to give a final solution pH of 6. This back extraction buffer was added to the top phase recovered from the forward extraction, in a mass ratio of top phase to back extraction buffer of 1:2. In order to test the observation from Andrews et al., that the interfacial precipitate formed during the forward extraction could be made to resolubilise during the back extraction process, two back extraction systems were set up. One in which the back extraction was performed on the top phase together with the interfacial precipitate from the forward extraction, and another in which the back extraction was performed only on the top phase (i.e. without the interfacial precipitate) from the forward extraction. The idea behind the back extraction step is to preferentially partition the mAb from the polymer phase back into a salt phase allowing for further processing. Table 6.3 summarises the results obtained.

The back extraction process resulted in the formation of an interfacial precipitate,

Table 6.3: Performance of back extraction systems

Back Extraction System	Partition Coefficient (K)	Phase Ratio (V_t/V_b)	mAb Yield Top Phase (Measured)	mAb Yield Bottom Phase (Measured)	mAb Yield Precipitate (Calculated)
With Interfacial Precipitate from Forward Extraction	8.78	0.5	6%	1%	93%
Without Interfacial Precipitate from Forward Extraction	21.79	0.5	14%	1%	84%

regardless of whether a precipitate was initially present or not. Performing a mass balance upon the back extraction system (assuming that the mAb concentration determined for the top phase from the forward extraction is correct), it was seen that a very small mAb yield was obtained in the bottom salt phases (1%). Furthermore, only a small amount of mAb remained in the top phase (between 6 and 14%). This indicates then that the remaining mAb must be present in the interfacial precipitate.

This observation presents a further divergence from the system performance as described by Andrews et al. There it was described that the interfacial precipitate formed during the forward extraction could be resolubilised during the back extraction. However in the case of this study, not only did the interfacial precipitate formed during the forward extraction not dissolve during the back extraction, but the back extraction process caused further precipitation to occur. Furthermore this precipitate seemingly contains the majority of the mAb which partitioned to the top phase during the forward extraction. The results (Table 6.3) also indicate that including the precipitate formed during the forward extraction can increase the amount of mAb precipitation during the back extraction.

mAb Precipitation Characterisation

Protein A analysis of the interfacial precipitate, following recovery and resolubilisation, revealed as expected a high mAb content, and also interestingly a relatively

low impurity composition. Thus whilst the back extraction did not behave in the manner in which it was expected, it did nonetheless result in partial purification of the product. The potential for exploiting this phenomenon for protein purification purposes was sufficient that an alternative *ATPE augmented precipitation process* was proposed, in which the dual bioseparation mechanisms utilised by ATPE and protein precipitation were combined. A patent application for this proposed process was submitted and is detailed in Chapter 12. However the aim of this study was evaluate ATPE, rather than any alternative form of the process. As a result, the observed precipitation during the back extraction, despite the potential processing benefits, was considered an unwanted deviation, and as a result was not considered for further incorporation into this particular study.

6.6.1 Decreased Phosphate Concentration

In light of the undesirable system performance described previously, attempts were made to try and further develop the back extraction process, in order to encourage mAb to partition to the bottom phase during this stage of the ATPE process. As described previously, the partitioning of a mAb in an ATPE system is influenced by a number of factors including a mixture of both electrostatic effects, and reduced solubility caused by the phase forming components. It was hypothesised that the precipitation of mAb at the interface of the back extraction two phase system was due to a combination of reduced electrostatic interactions with the top phase and a low level of mAb solubility in the bottom phase. The reduced electrostatic interactions with the top phase are a result of their being no NaCl added in the back extraction system. The only NaCl present in the back extraction system is that which is carried over with the top phase from the forward extraction. Chloride ions, when introduced to a PEG - Phosphate aqueous two phase system will partition evenly between the top and bottom phases. During the forward extraction, electrostatic interactions between the negatively charged chloride ions in the top phase and the positively charge mAb, along with a reduction in the overall protein solubility in the bottom phase due to its high ionic strength, cause the mAb to partition preferentially into the polymer rich

phase. In the back extraction, the reduced level of chloride ions in the aqueous two phase system should result in the reverse behaviour. A lower chloride concentration in the system should cause a decrease in the level of electrostatic interactions with the top phase. Without these interactions, the top phase, which contains a relatively high concentration of water binding PEG molecules, becomes no longer a thermodynamically favourable phase for the mAb to remain within. Meanwhile, a reduction in the system chloride concentration will increase the protein solubility in the bottom phase. Presented with these two options, the mAb should theoretically preferentially partition into the bottom phase.

The fact that the mAb precipitates at the interface between the top and bottom phase indicates that only half of the conditions required during the back extraction for the mAb to partition into the bottom phase have been met. Clearly, the loss of electrostatic interactions with the top phase, coupled with the relatively high concentrations of PEG, has resulted in the mAb moving out of this phase. However the reduction in the ionic strength of the bottom phase seems not to have been sufficient in order to facilitate the partitioning of mAb to this phase. Thus faced with two aqueous phases containing high concentrations of precipitating agents (PEG and phosphate), the mAb comes out of solution and settles at the interface between them. The ATPE back extraction system then has essentially become a precipitation process.

In order to remedy the situation, the back extraction system was modified so as to contain lower concentrations of phosphate. It was hoped that this would provide a bottom phase with an environment more amenable for the mAb partitioning. Several different back extraction systems were tested, with the phosphate concentration reduced from 17% (w/w) to 12.4% (w/w), 11.2% (w/w), 10.0% (w/w), 8.7% (w/w) and 7.5% (w/w). In addition, the volumetric ratio between the top phase from the forward extraction and the back extraction buffer added was reduced from 1:2, to 1: 1.2 which mimics the phase ratio observed between the top and bottom phases during the forward extraction. In the original version of the back extraction process, the bottom phase of the back extraction system was observed to be twice that of the top phase from the forward extraction (Table 6.3). If the mAb were to partition to

the bottom phase, at this phase ratio, then this step would effectively be diluting the product, after the forward extraction step has allowed a concentration of the mAb to be achieved. Since product concentration is a key aim for any bioseparation technique to be employed early on in a downstream process, the volume of back extraction buffer was reduced in order balance the volumetric ratio between the top and bottom phases of the back extraction system. The effects of reducing the phosphate concentration on the partitioning of mAbs in the back extraction are summarised in Table 6.4.

Table 6.4: Impact of reducing the phosphate concentration in back extraction systems

Back Extraction System Phosphate Concentration (%w/w)	Partition Coefficient (K)	Phase Ratio (V_t/V_b)	mAb Yield Top Phase (Measured)	mAb Yield Bottom Phase (Measured)	mAb Yield Precipitate (Calculated)
12.5%	35.13	1.19	25.2%	0.6%	74.2%
11.2%	21.27	0.80	14.6%	0.9%	84.6%
10.0%	34.59	1.40	30.5%	0.6%	68.9%
8.7%	34.65	2.60	56.4%	0.6%	42.9%
7.5%	26.24	5.00	162.7%	1.2%	0.0%

Lowering the phosphate concentration of the two phase system along with reducing the volumetric ratio between the top phase and the phosphate buffer did have an impact upon the partitioning behaviour of mAb in the back extraction system. Table 6.4 shows an increase in mAb yield in the bottom phase with decreasing phosphate concentration, however even at a system phosphate concentration of 7% (w/w), the mAb yield is still only just over 1%, making it comparable with the bottom phase yields achieved under the original back extraction conditions. From this perspective, reducing the volume ratio of back extraction buffer added, along with the system phosphate concentration did not result in a significant improvement in performance. These changes however had a significant impact upon the mAb yield in the top phase of the back extraction system. Table 6.4 shows that the mAb yield in the top phase of the back extraction system increases with decreasing phosphate concentration. This is most likely caused by the operating point on the PEG phosphate phase

diagram, moving to a lower tie-line on the binodial curve (Figure 6.5) as the phosphate concentration is reduced, resulting in a top phase with a lower PEG concentration, thereby increasing the protein solubility in this phase.

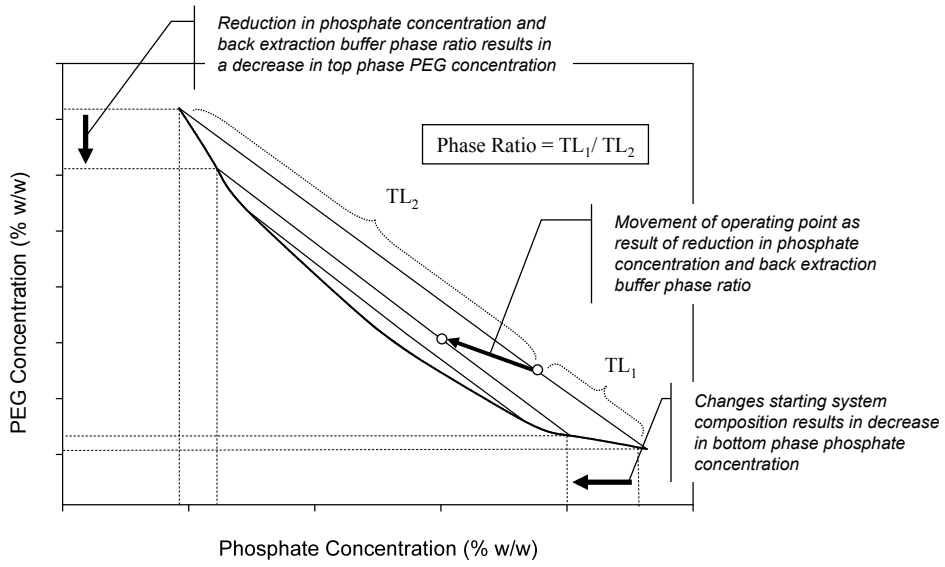


Figure 6.5: Schematic showing impact of changes in aqueous two phase back extraction system composition in terms of the binodial phase diagram. Conditions above the binodial curve result in two phase formation whilst those below do not. The x-axis represents the concentration of either Phosphate, or citrate salt used in the two phase system

Whilst interfacial precipitates formed in all of these "lower phosphate" back extraction systems, the actual amount of precipitation observed decreased with decreasing phosphate concentration. Indeed the back extraction system comprising 7% (w/w) phosphate was observed to contain only negligible amounts of interfacial precipitate. Correspondingly, the amount of mAb in the top phases implies that the yield loss in this precipitate decreases with decreasing phosphate concentration which may be seen as a beneficial outcome. Indeed a mass balance of the back extraction system containing 7% phosphate showed no mAb present in the interfacial precipitate. The exact mAb concentration of this precipitate was difficult to determine due to the

previously described effect, in which mAb concentrations of samples containing PEG are overestimated by the assay method used. As a result for the 7% phosphate back extraction system, the top phase mAb yield was determined to be greater than 100%. Regardless, the high top phase yield, coupled with the low bottom phase yield, and also the relatively small amount of precipitation which was actually observed indicates that the level of mAb lost through precipitation in this back extraction system is at least very low.

From the trend seen in Table 6.4, it could be presumed that the product yield in the bottom phase could be increased by reducing the phosphate concentration further to below 7% (w/w), however it was found that at phosphate compositions lower than this, there was no two phase formation with conditions in the system dropping below the binodial curve of the PEG - Phosphate phase diagram. Based on these results, whilst it seems that reducing the phosphate concentration caused a reduction in the level of mAb lost through precipitation, it has achieved this by increasing the protein solubility in the polymer rich top phase. As a result the mAb has partitioned into the top phase of the back extraction system rather than the bottom phase where it is desired. Thus whilst the performance of this modified back extraction system may be considered an improvement over that given by the original system, due to a reduction in the level of precipitation, it is still far from ideal.

6.6.2 Decreased System pH

In light of the effects seen with reducing the phosphate concentration, attempts were made to further develop the back extraction system in order to increase the mAb yield in the bottom phase. Reducing the volume ratio of back extraction buffer used and reducing the overall phosphate concentration of the system was seen to cause a decrease in mAb precipitation and an increase in mAb partitioning to the top phase. Based on these observations, it may be concluded that the changes which were made to the back extraction system essentially increased the mAb protein solubility in the top phase preventing it from precipitating at the interface. Thus as is the case with the forward extraction system, it seems that it is more thermodynamically favourable

for mAbs to partition to the polymer rich top phase rather than the salt rich bottom phase of the back extraction system, where it is desired. Efforts were therefore made in order to try and increase the thermodynamic attractiveness of the bottom phase.

As previously discussed, a major driving force causing mAbs to partition into the bottom phase of a PEG - phosphate aqueous two phase system, are the electrostatic interactions between positively charge mAb molecules and negatively charged phosphate anions. To this point, all ATPE systems had been operated at a pH of 6.0, which is below the pI of the antibody used thereby ensuring that the mAb molecules carry a net positive charge. This charge should theoretically cause the mAbs to preferentially partition to the bottom phase of the back extraction system. The fact that they do not implies that perhaps the size of the net charge carried by these mAbs is not large enough to cause the desired partitioning behaviour. As a result, it was postulated that a decrease in system pH would increase the size of the net positive charge carried by the mAb molecules, which in turn would make it more thermodynamically favourable for the mAb to partition into the phosphate rich bottom phase where the mAbs could form electrostatic interactions with the strongly hydrated anion.

An additional effect of reducing the pH and increasing the positive charge carried by the mAb molecules could be that it may also help lower the level of mAb precipitation which had been observed in the majority of back extraction conditions tested. Indeed, to this point, all except one of the back extraction systems (containing only 7% (w/w) phosphate) resulted in a significant degree of mAb yield loss through precipitation. Increasing the size of the net positive charge by lowering the pH should increase like charge repulsion forces between mAb molecules, thereby helping to reduce the level of precipitation.

Back extraction systems containing the same concentrations as shown in Table 6.4 were generated, with the pH of the back extraction buffer used lowered from pH 6.0 as was previously the case to pH 3.2. The results are summarised in Table 6.5.

Lowering the pH of the back extraction system had a number of dramatic effects. Firstly no interfacial precipitate was formed in any of the back extraction systems. Instead, it seems that the mAb has almost exclusively partitioned to the top polymer

Table 6.5: Impact of reducing back extraction system pH

Back Extraction System Phosphate Concentration (%w/w)	Partition Coefficient (K)	Phase Ratio (V_t/V_b)	mAb Yield Top Phase (Measured)	mAb Yield Bottom Phase (Measured)	mAb Yield Precipitate (Calculated)
12.5%	726.06	1.00	161.3%	0.2%	0.0%
11.2%	250.72	1.22	161.6%	0.5%	0.0%

rich phase, with a less that 1% yield of mAb in the bottom phase. Also two phase systems did not form at phosphate system concentrations lower than 11.2% (w/w). This indicates that lowering the pH, caused a shift in the binodial curve such that operating points of 10% (w/w) and lower are beyond the conditions that would cause two phase formation.

The lack of interfacial precipitate implies a number of things. Firstly it may well be that as predicted the increased net positive charge of the mAb molecules, and the resultant like charge repulsion has prevented mAbs molecules from interacting with one another to form any precipitation. As a result, whereas pH 6.0 back extraction systems with 12.5% (w/w) and 11.2% (w/w) phosphate, caused significant levels of mAb precipitation, systems with the same composition, albeit at a lower pH did not contain any precipitated mAb. Previous results however showed that even in systems in which mass balances showed no mAb loss due to precipitation, an interfacial precipitate was still present nonetheless, albeit in very small amounts. This indicates that the interfacial precipitate formed in all of the pH 6.0 back extraction systems is composed of more than just mAbs, but must also contain at least one unknown process stream impurity. However the fact that no precipitate is formed at all in the pH 3.2 back extraction system indicates that not only has the lowered pH reduced the level of mAb precipitation, but it has also reduced the level of precipitation of the unknown impurity.

The most significant observation from this set of experiments was that even with a lowered system pH during the back extraction, the mAb still remains exclusively

in the top phase. Thus whilst issues of yield loss through mAb precipitation seemed to have been resolved by lowering the system pH, the desired partitioning of mAb to the bottom phase was still an elusive outcome.

6.6.3 Alternative Anion

The phosphate rich bottom phase of the back extraction system clearly represents a thermodynamically unfavourable phase for the mAb to reside within. As a result even at a lowered system pH, where electrostatic interactions between the positively charged mAb and the negatively charged phosphate anions would be high, the mAb still remains in the polymer rich phase. It was therefore felt that it may indeed be the phosphate anions themselves which are limiting the movement of the mAb into the salt rich bottom phase.

PEG polymers are known to form aqueous two phase systems with a wide range of kosmotropic salts including phosphates, citrates and sulphates. In this respect, the key component of these salts are the negatively charge anions. The Hofmeister series, summarised in Figure 6.6, ranks anions in order of their relative abilities to alter the network of hydrogen bonding in water. Anions towards the left hand side of Cl^- are known as kosmotropes. These anions are strongly hydrated, reducing protein solubility, causing salting-out effects and by extension protein precipitation. Anions to the right hand side of Cl^- are known as chaotropes. These anions help increase protein solubility through salting-in effects. The inequality symbols in Figure 6.6 therefore indicate the relative abilities of these anions to cause precipitation, and specifically in the original studies performed by Hofmeister, the precipitation of serum globulins.

Sulphate (SO_4^{2-}), phosphate (PO_4^{3-}) and citrate ($C_3H_5O(COO)_3^{3-}$), are to the far left of the Hofmeister series and their relative ranking implies that lower concentrations of sulphate than phosphate and lower concentrations of phosphate than citrate are required to cause the salting-out and precipitation of a given amount of protein in solution.[113] Therefore, of the three possible kosmotropes, citrate rather than phosphate would be a more logical choice for the anion of the bottom phase salt, since it is the least hydrating and thus at a particular concentration, would provide the highest

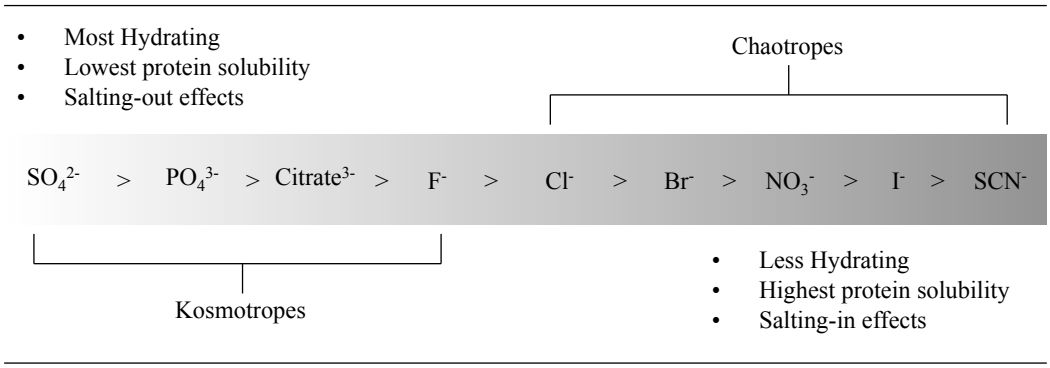


Figure 6.6: The Hofmeister Series

protein solubility. Based on this rationale, the series of back extraction experiments were repeated, but with the phosphate back extraction buffer replaced by a concentrated citrate solution. By having a citrate, rather than phosphate, based bottom phase it was hoped that the product mAb could be presented with a more attractive salt rich phase in which to partition during the back extraction.

The forward extraction was still performed using phosphate. The top phase from this forward extraction was however contacted with a concentrated citrate solution, containing 30% (w/w), 27% (w/w), 24% (w/w), 21% (w/w) and 18% (w/w) sodium citrate all at a pH of 4.7. The volume ratio between the top phase from the forward extraction and the citrate back extraction buffer was maintained at 1:1.2, giving back extraction systems with overall citrate concentrations of approximately 18% (w/w), 16% (w/w), 14% (w/w), 12.5% (w/w) and 11% (w/w) respectively. The results are summarised in Table 6.6

The substitution of phosphate with citrate in the back extraction did improve the partitioning of mAb to the bottom phase to a certain extent. At a citrate system composition of 18% (w/w), a bottom phase mAb yield of 2% was achieved, whilst a citrate concentration of 16% (w/w) resulted in a bottom phase mAb yield of 8%. At citrate concentrations lower than 16%, no two phase formation was observed. The higher concentrations of citrate required for two phase formation (two phase formation was still observed at phosphate concentrations of 11%) may be reflective of the move

Table 6.6: Impact of Using Citrate in Place of Phosphate for Back Extraction

Citrate Composition of Back Extraction System (%w/w)	Partition Coefficient (K)	Phase Ratio (V_t/V_b)	mAb Yield Top Phase (Measured)	mAb Yield Bottom Phase (Measured)
18%	967.18	0.81	161.0%	1.7%
16%	23.41	0.81	156.0%	8.2%
14%				
12.5%		No Two Phase Formation		
11%				

further down the Hofmeister series since the formation of the two phase system may be thought of as being a form of salting-out of the PEG polymer. The phase ratio in the citrate based back extraction system was found to be 0.81, which is lower than that of the phosphate back extraction system and is comparable to that observed in the forward extraction.

The use of citrate in place of phosphate clearly had the desired effect of improving the partitioning of mAb to the bottom phase during the back extraction. This is presumably a result of increasing the protein solubility of the salt rich bottom phase of the back extraction process. The mAb yield of 8%, obtained using a citrate concentration of 16% (w/w) is a significant improvement over the 1% yields obtained using the phosphate based back extraction system.

Lowering the citrate concentration beyond 16% (w/w), would inevitably increase the yield beyond 8%, as the protein solubility in the bottom phase increases even further. The point that citrate concentrations below 16% are not possible raises an interesting point regarding the development of the back extraction process. On the one hand lowering the concentration of the citrate in the back extraction system definitely results in an increase in the protein solubility of this phase. At the same time however the same aqueous processes which are responsible for protein solubility also presumably influence the formation of the two phase system. Hofmeister, specific ion, effects are known to be ubiquitous and are a heady mix of electrostatic interactions

between the different components of an aqueous system.[114] Increasing one system characteristic (protein solubility), seems to diminish the other (tendency for aqueous two phase formation). The situation is further complicated by the fact that partitioning of mAbs to the bottom phase goes beyond just protein solubility effects, as electrostatic interactions between the mAb and the anions in the bottom phase also play a part. Citrate anions have a lower charge density than phosphate (as represented by the Hofmeister series). Thus, for mAb molecules in an aqueous two phase back extraction system, the electrostatic attraction of a bottom phase consisting of citrate anions would be smaller than that of a phosphate based bottom phase. As a result, whilst the mAb may have a higher solubility in the citrate bottom phase, the electrostatic driving force for partitioning there is diminished when compared to a bottom phase containing phosphate anions. On the other hand, whilst the phosphate anions may impose a greater electrostatic attraction for the mAb, the solubility in this phase is severely compromised, leading to the observed high partition coefficients in the back extraction system. Thus whilst the use of citrate in place of phosphate in the back extraction resulted in an increase in mAb yield, limitations in terms of feasible concentrations for two phase formation and diminished electrostatic interactions with mAb mean that the overall mAb yield was still at a far from acceptable level.

6.6.4 Increasing PEG Concentration and Multi-stage Back Extraction

The use of citrate in place of phosphate for the back extraction process, was found to cause an increase in the level of mAb partitioning to the bottom phase. However it was found that the increased protein solubility in the bottom phase also came at a price, namely a requirement for higher anion concentrations in the back extraction system and also a reduction in electrostatic interactions, ultimately limiting the overall product yield in the bottom phase.

An additional factor which must be considered is the link between the overall system composition and that of the individual top and bottom phases. Because

the aqueous two phase system is a closed static equilibrium, changes to one phase inevitably have an impact on the other. Figure 6.7 shows the movement of the operating point across the PEG-citrate phase diagram as a result of decreasing the citrate concentration of the back extraction system (point 1 to 2 to 3). From this schematic, it can be seen that as the citrate concentration is lowered there is a concomitant decrease in the citrate concentration of the bottom phase (arrow D) which presumably leads to an increase in protein solubility and thus an increase in the mAb yield in the bottom phase. At the same time there is also an increase in the citrate concentration in the top phase (arrow B) which, consistent with the same logic, reduces the protein solubility in the top phase, thus providing an extra driving force for movement of mAb into the bottom phase. Interestingly, Figure 6 also shows that as the citrate concentration of the back extraction system is reduced, the PEG concentration in the Top Phase decreases (arrow A), whilst the concentration in the bottom phase actually increases (arrow C). As is the case with citrate, due to its water binding nature, the presence of PEG in any phase will serve to reduce the protein solubility. Therefore as the operating point moves from position 1 to 3 on the phase diagram, the protein solubility in the bottom phase is actually being decreased by the partitioning of PEG in the aqueous two phase system. Thus whilst lowering the citrate concentration of the back extraction system increases the protein solubility in the bottom phase in terms of the movement of citrate anions, the opposite effect is being caused by the movement of the PEG molecules.

Based on the results observed the solubility of the mAb molecules seem to be more greatly affected by the kosmotropic anion of the two phase system than the polymer. This is most clearly seen by the fact that mAb remains predominantly in the top phase of both the forward and back extraction system. Thus is may be argued that the increased mAb protein solubility in the bottom phase as a result of decreased citrate concentration outweighs any loss of solubility as a result increasing PEG concentration.

Given the previously observed results, one potential option considered was to move to a salt lower in the Hofmeister series such as tartrate, which may increase the

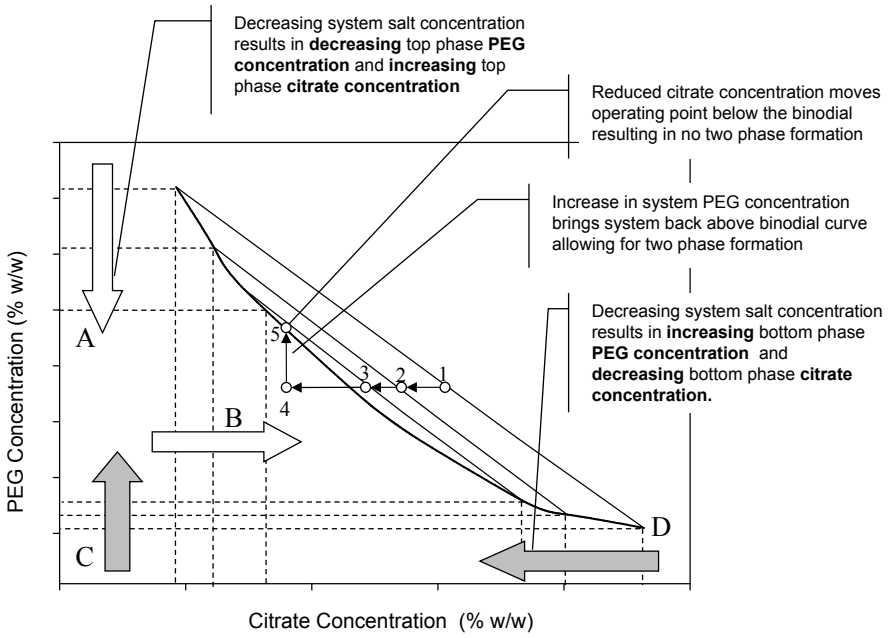


Figure 6.7: Schematic showing movement of operating conditions on the PEG / Citrate phase diagram. As the citrate concentration is reduced, the top phase PEG concentration decreases whilst the citrate concentration increase. The reverse is true of the bottom phase composition.

protein solubility, but the reduction in charge density would presumably decrease the electrostatic attraction of the salt rich phase. In addition to this, use of a salt lower in the Hofmeister series will most likely require higher concentrations in order to ensure two phase formation, thereby partially counteracting any benefit gained by using a less hydrated anion. The results which had been observed to this point indicated that further reductions in the citrate concentration would lead to greater mAb solubility in the bottom phase and decreased solubility in the top phase. However, as detailed previously lowering the citrate concentration of the back extraction system below 16% (w/w) resulted in no two phase formation. This is because the operating point is moved past the binodial curve into the one phase region of the phase diagram, shown by Point 4 in Figure 6.7. The only way to generate a two phase system at such a relatively low concentration of citrate would be to increase the PEG concentration of the back extraction system, thereby moving the operating point from position 4 to position 5 in Figure 6.7.

A new back extraction was therefore performed, this time by injecting a concentrated shot of 50% (w/w) PEG into a back extraction system formed from the top phase of the forward extraction and a 30% (w/w) Sodium Citrate back extraction buffer, added to give a final system citrate concentration of 13% (w/w). The size of the concentrated PEG shot was approximately 14% (v/v) of the total system volume. This resulted in the formation of an aqueous two phase system with a phase ratio of 9, and a partition coefficient of 15. The mAb yield in the bottom phase was found to be only 1%. Thus the strategy of further increasing the mAb solubility of the bottom phase, by decreasing the citrate concentration was found to be ineffective. There are a couple of factors which may help explain the results of this back extraction.

Firstly, as illustrated in Figure 6.7, reducing the citrate concentration of the back extraction has the result of increasing the PEG concentration of the bottom phase. In the back extraction systems utilising 18% (w/w) and 16% (w/w) citrate, the loss of mAb protein solubility as a result of increasing PEG concentration was seemingly outweighed by the gain in solubility caused by the reduction in the citrate concentration of the system. Assuming that the tie-line on which the operating point represented

by the back extraction system with a citrate concentration of 13% (w/w) lies, is below that of the corresponding tie line for the back extraction system with a citrate concentration of 16% (w/w), then the PEG concentration in the bottom phase of the former system will be higher than that of the latter (essentially assuming that Point 5 in the schematic phase diagram shown in Figure 6.7 really does lie upon a tie line below that of Point 3). It therefore seems that with the 13% (w/w) citrate back extraction system the loss of protein solubility in the bottom phase, as a result of the increase in PEG concentration now outweighs any benefit gained by reducing the citrate concentration. The situation has therefore in a sensed flipped, such that the influence upon mAb solubility imposed by the PEG present in the bottom phase is greater than that of influence imposed by the citrate anions. As a result, whereas the back extraction system with a citrate concentration of 16% (w/w) had a bottom phase mAb yield of 8%, the back extraction system with a citrate concentration of only 13% (w/w) has a bottom phase yield of merely 1%.

Another factor that must be taken into account is the phase ratio, which in the case of the 13% (w/w) citrate back extract system is 9, compared to the phase ratio of 0.8 observed for the back extraction system with a citrate concentration of 16% (w/w). This means that the top phase has a volume nine times greater than that of the bottom phase. Considering just the masses of material involved, it seems that the yield of mAb is being partially limited by the volume of bottom phase into which it may partition. Indeed the partition coefficient (K) of the 13% (w/w) citrate back extraction system is actually lower than that of the 16% (w/w) system (K = 15 compared to K = 24 respectively). This means that the bottom phase of the 13% (w/w) system has a greater affinity for mAb than that of the 16% (w/w) system. However because the actual volume of the bottom phase is so small, the overall yield of the 13% (w/w) citrate system is lower. This is analogous to a chromatography scenario in which two Protein A columns, X and Y, are being used to capture mAb from cell culture supernatant. Column X has a dynamic binding capacity of 30g/L and a volume of 3L, whilst column Y has a dynamic binding capacity of 50g/L and a volume of 1L. Column X can be used to capture 90g of mAb whilst column Y, even

though it has a higher binding capacity, can only be used to capture 50g of product.

One action which may be taken is to move to another operating point, further down the same tie-line in order to alter the phase ratio and increase the relative volume of the bottom phase. This could be done by increasing the volume of the back extraction buffer added to simultaneously increase the citrate concentration and decrease the PEG concentration of the back extraction system. However doing so, without moving the operating point to an undesired tie-line, such as one which has already been investigated would be difficult without complete characterisation of the PEG-Citrate phase diagram, something which was deemed to be beyond the scope of this present study. Regardless, the partition coefficient displayed by the back extraction system with a citrate concentration of 13% (w/w) is significantly greater than 1, with a K-value of 15. Ideally the K value would be closer to the reciprocal of this. Thus even if an operating point further down the tie line could be found, the concentration of mAb in the bottom phase is such that with these concentrations it is still unlikely that the overall yields would be at a level which could be deemed acceptable.

It was felt at this stage of the ATPE process development that a threshold had been reached in terms of manipulating the concentrations of PEG and citrate of the back extraction system in order to maximise mAb partitioning to the bottom salt rich phase. Thus an alternative approach was considered. Aside from PEG, phosphate and citrate another component of the ATPE systems under investigation is NaCl. Sodium chloride is responsible for providing favourable electrostatic interactions between the mAb and the top phase, and also responsible for increasing the ionic strength of the bottom phase in order to reduce the protein solubility therein. In doing this NaCl effectively acts as a partitioning facilitator, providing a driving force for the partitioning of mAbs into the top phase. As has been determined in a previous study[110], without the presence of NaCl in the forward extraction system, mAbs will tend to partition into the salt rich bottom phase.

Whilst no NaCl is added to the back extraction system, some Na^+ and Cl^- ions will have been carried over from the first forward extraction. Based on the results

which have been observed, with the top phase of all the back extraction systems investigated still having a high affinity for the mAb, it is evident that the amount of NaCl present in the back extraction system is still sufficiently high as to provide the same partitioning influence as it did in the forward extraction. Based on this logic, it was thought that decreasing the NaCl concentration of the back extraction system would result in an increase level of mAb partitioning to the bottom phase. The difficulty however is that the PEG and NaCl present in the back extraction system originate exclusively from the top phase from the forward extraction. Diluting the NaCl to lower the concentration would also lower the concentration of the PEG which is not desired. As a result a different strategy was devised, making use of the fact that NaCl partitions equally between top and bottom phase of a PEG phosphate/ citrate aqueous two phase system. The decision was taken to attempt to perform a multi-stage back extraction.

The forward extraction contains 12% (w/w) NaCl. During the forward extraction process, this NaCl partitions equally between top and bottom phases. Thus half of the NaCl present in the forward extraction system will be present in the back extraction. Assuming a simplified phase ratio of 1 (the actual phase ratio of the forward extraction being 1.2) this will mean that once the back extraction buffer is added, the total NaCl concentration in the back extraction system will be approximately 6% (w/w). Based on the observed results, a NaCl concentration of 6% (w/w) is still sufficiently high so as to encourage mAb partitioning to the top phase of the back extraction process. If the top phase from this back extraction, which contains the majority of the mAb were to be subjected to a second back extraction, and again assuming a simplified phase ratio of 1, then the NaCl concentration of this second back extraction system would be approximately 3% (w/w). If the top phase from this back extraction step were to be recovered and subjected to another back extraction process, then making the same assumptions, the NaCl concentration in this third system would be only 1.5% (w/w). The sequential back extraction steps would allow the NaCl concentration to be reduced without significant changes in the PEG concentration of the back extraction system. The PEG concentration would inevitably

decrease since some polymer will partition into the bottom phase of both the forward extraction and the first back extraction. Likewise, whilst the aim is to add sufficient phosphate/ citrate in each back extraction to match the initial concentration in the forward extraction system, the amount of phosphate in the system will also steadily increase since there will be some phosphate/ citrate present in the top phase from the forward and first back extraction. The movement of the operating point about the phase diagram is illustrated schematically in Figure 6.8.

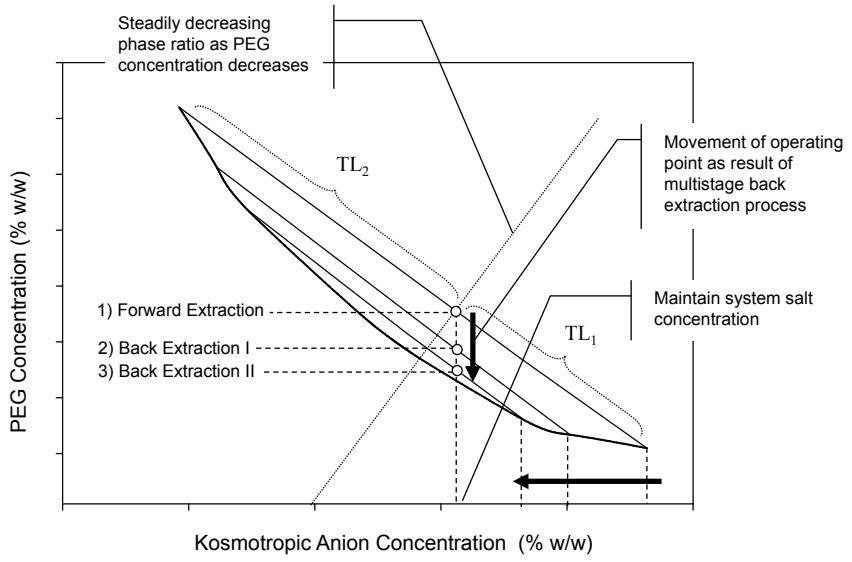


Figure 6.8: Schematic showing movement of operating point about the PEG / Citrate phase diagram during multi-stage back extraction

Performing a multistage back extraction will also cause a decrease in the phosphate/ citrate concentration of the bottom phase of each subsequent back extraction step. Based on this rationale, the bottom phase of Back Extraction II and III as shown in Figure 6.8 should have a higher mAb yield than any previously investigated back extraction system.

An experiment was therefore performed in which the forward extraction was performed as previously described, with a composition of 15% (w/w) PEG, 14% (w/w)

phosphate and 12% (w/w) NaCl. A back extraction was then performed in which a concentrated 30% (w/w) Na-citrate back extraction buffer was added to the top phase recovered from the forward extraction, in a volume ratio, top phase to extraction buffer, of 1:1.2 to give a final system concentration of 16% (w/w) Na-citrate. The top phase from this back extraction was then recovered and then subjected to a further back extraction step, by contact again with fresh 30% (w/w) Na-citrate back extraction buffer, in a volume ratio of 1:1.2 to give a final system concentration of 16% (w/w) Na-citrate. The top phase from this second back extraction was then subjected to a third and final back extraction in which it was further contacted with fresh back extraction buffer with the same concentration and volume ratio as in each of the previous back extractions.

The results of this experiment are summarised in Table 6.7.

Table 6.7: Results of multi-stage back extraction

Step	Partition Coefficient (K)	Phase Ratio (V_t/V_b)	mAb Yield Top Phase (Measured)	mAb Yield Bottom Phase (Measured)	System NaCl Concentration (%w/w)
Forward Extraction	165.33	1.00	138.5%	0.8%	8%
Back Extraction I	209.66	0.81	166.6%	0.7%	3%
Back Extraction II	8.26	0.20	76.94%	46.5%	1%
Back Extraction III	0.00	0.03	0.00%	17.7%	1%

The decrease in the NaCl composition of subsequent back extraction systems had the predicted effect upon the partitioning of the mAb. The mAb yield in the bottom phase after the first back extraction step (Back Extraction I) was as had been previously observed, very low, at less than 1%. However when the top phase of this back extraction was subjected to an additional back extraction step (Back Extraction II), the bottom phase mAb yield increased to 46.5%. This is a significantly larger yield than had been achieved with any of the previously detailed back extraction systems. Subjecting the top phase from Back Extraction II to a third and final back extraction step (Back Extraction III), resulted in a bottom phase mAb yield of 17.7%, giving

the total mAb yield, recovered in the bottom phase from each back extraction, of approximately 64%.

It should be noted at this point that whilst the overall process yield of 64%, achieved using the multi-stage back extraction approach, may seem modest, it must taken in context with the mAb yield obtained using Protein A chromatography. The aim of this study was to ultimately test the impact of using ATPE in place of Protein A in the mAb purification platform process (Figure 6.2).

Thus it should ideally be possible to match the yield which may be obtained using the more conventional process option. Whilst Protein A will typically be able to provide mAb yields of around 98%, as discussed previously, the yield obtained in this study was actually only 69% (Table 6.1). This low yield value was attributed to an overestimation of the mAb feed concentration. Assuming that similar overestimations do not apply to the determination of mAb concentrations in the bottom phase of these back extraction systems, then the 64% yield achieved using the ATPE process suddenly becomes significantly more interesting. In fact if it is assumed that the 69% yield obtained from Protein A, actually represents a yield of 98%, once feed overestimations are accounted for, then extrapolation of the results would imply that the yield achieved from this ATPE process is in reality closer to 91%.

In the final back extraction (Back Extraction III), the two phase system which was formed had a very negligible top phase volume, thus giving the very small phase ratio of 0.03. The steady drop in phase ratio may be explained using Figure 6.8, where it can be seen, that subsequent back extraction steps cause the operating point to move to lower regions of each successive tie-line. The mAb concentration in this top phase was found to be insignificant. An interfacial precipitate was observed in the final Back Extraction III system, which was not present in any of the previous two back extraction systems, or the forward extraction system for that matter. It was assumed that this precipitate contained any remaining mAb which was not found to be present in the recovered bottom phases, accounting for any potential overestimation of the mAb feed concentration.

The strategy of decreasing the NaCl concentration therefore had the desired effect

of increasing the mAb yield recovered in the bottom phase of the back extraction system. Indeed, if it is assumed that the feed concentration has been overestimated and that these same overestimations do not stretch to cover the analysis of the bottom phases of these aqueous two phase systems, then a yield of 64% may be considered highly acceptable. However the use of this multi-stage back extraction does have several significant drawbacks, particularly when considering the operation of such a scheme at a large scale. Of major concern is the issue of product dilution. The results shown in Table 6.7 are from an experiment performed on a 30g ATPE system. The initial cell culture supernatant feed volume to this system was 17.8mL. The volume of bottom phase obtained of Back Extraction II was approximately 26mL, whilst the volume of bottom phase obtained from Back Extraction III was approximately 11mL, giving a total product pool volume of 37mL. This is more than twice the volume of the original feed. Therefore the entire ATPE process has resulted in a dilution of the product rather than a concentration. This is a far from desirable, particularly considering the fact that the relatively low resolving power of ATPE makes it suited only for application at an early point in the process train where product concentration is a key objective. This issue of product dilution may be directly attributed to the associated partition coefficients of the back extraction systems. The majority of mAb is recovered during the second back extraction step, however the partition coefficient during this step was found to be significantly greater than unity, which means that the concentration of mAb in the top phase is higher than that in the bottom. This troublingly means that the top phase still remains more attractive than the bottom phase, even when the NaCl concentration of the bottom phase is reduced. Thus whilst the yield achievable using the ATPE process has been made to reach an acceptable level, the dilution of the product due to relatively high partition coefficients of the back extraction systems remains a problem.

6.6.5 Decreasing NaCl Concentration

It was felt that with the multi-stage back extraction and the gradual reduction in NaCl concentration, almost all options of increasing the level of partitioning of mAb to the

bottom phase of the ATPE back extraction systems had been exhausted. As stated previously the presence of NaCl in this ATPE system is a major driving force for the partitioning of mAb into the polymer rich top phase. Its elimination from the system should therefore encourage mAb movement into the bottom phase, yet even with an overall system NaCl concentration of 2% (w/w), the top phase still seemingly has a higher affinity for the product antibody than the salt rich bottom phase. To further investigate this, an array of ATPE forward extraction experiments were performed, containing NaCl concentrations of 10% (w/w), 8% (w/w), 6% (w/w), 4% (w/w) and 2% (w/w), as opposed to the 12% originally defined by Andrews et al. The results are summarised in Table 6.8.

Table 6.8: Results of Decreasing NaCl Concentration during Forward Extraction

NaCl Composition of Forward Extraction System (%w/w)	Partition Coefficient (K)	Phase Ratio (V_t/V_b)	mAb Yield Top Phase (Measured)	mAb Yield Bottom Phase (Measured)
10%	175.33	0.89	156.5%	1.0%
8%	337.50	0.89	136.4%	0.5%
6%	331.24	0.89	107.8%	0.4%
4%	300.00	0.89	85.5%	0.3%
2%	233.33	0.89	64.6%	0.3%

In all systems, the mAb yield in the bottom phase was found to be less than 1%. In addition to this, it seemed that the NaCl present in the forward extraction system somehow serves to maintain the product mAb in solution. At NaCl concentrations of 6% and lower, a significant amount of precipitation was observed following the addition of PEG to the system (powders were added sequentially in the order NaCl - PEG - Phosphate). This was accompanied by a drop in the top phase mAb yield following two phase formation. Thus not only does the removal of NaCl cause no increase in the mAb partitioning to the bottom phase, but it also seems to reduce the overall solubility of the product, leading to the observed precipitation. Comparing this to the results obtained by Andrews et al., where mAb was found to partition

almost exclusively to the bottom phase in systems containing no NaCl, it must be concluded that some specific property or properties of the cell culture supernatant feed being used in this study is limiting the affinity of the salt rich phases of these PEG - Phosphate/ Citrate ATPE systems for the product mAb.

These properties may be directly attributable to the mAb molecule itself. Movement of mAb into the bottom phase is facilitated by electrostatic interactions between the net positive charge carried by the mAb molecule at a physiological pH and the kosmotropic anions present in the bottom phase. However it may well be that the mAb molecules, whilst being positively charged, actually have large hydrophobic patches upon their surface, in which case interactions with PEG polymer molecules may be more thermodynamically favourable than with the charged anions. The poor partitioning of mAb into the salt rich phases of these systems may also be somehow related to the concentration of mAb in the cell culture supernatant feed. In the study by Andrews et al., the mAb titre in the feed used was only 1g/L, however in this study, the initial mAb feed concentration was almost 5 times this at 4.5 g/L. This difference in concentration may be influencing the partitioning behaviour of this ATPE system. Alternatively there may be some other component(s) present in the cell culture supernatant which is limiting the partitioning of mAb into the salt rich phase such as kosmotropic salts. Overall it may well be that a combination of all of these factors is in play.

Regardless, given that all rational approaches to developing the ATPE system in order to increase the mAb partitioning into the salt rich phase had failed, it was assumed that such behaviour was due to the characteristics of the feed material itself, and as such further attempts to reduce the partition coefficients by manipulating the component concentrations in the back extraction systems were at this point abandoned. Instead, efforts were refocused on attempting to modify the multi-step back extraction process in order to reduce the number of stages required. The multi-stage back extraction process described previously involved the use of three separate back extraction systems. This not only significantly increases the capital and operating costs associated with the overall ATPE process, if it were to be used in a large scale

manufacturing process, but also since the product is diluted during each back extraction step, the product concentration achieved during the forward extraction becomes severely compromised. Dilution of the mAb product can have serious implications upon the remaining unit operations in the downstream purification train. For example, if the ATPE process is to be followed by a packed bed chromatography operation, then increasing the process volume will lead to a concomitant increase in the loading time required for this subsequent step, which can have a deleterious effect upon the overall process productivity. For example if 10,000L of cell culture supernatant, containing 5g/L mAb is loaded onto a Protein A column with a dynamic binding capacity of 50g/L, then assuming that the bound product may be eluted in 3 column volumes, the final product pool will have a volume of 3000L and a concentration of approximately 16g/L. This product pool is then loaded onto a IEX chromatography column, with a dynamic binding capacity of 100g/L. Assuming the IEX column has a diameter of 200cm and the flow through the column is operated at a superficial fluid velocity of 200cm/h, then the total time required to complete the chromatography cycle can be expected to be anywhere in the region of 2 - 3 hours, depending upon the number of column volumes of buffer required for column equilibration and washing etc. If the same 10,000L of cell culture supernatant were processed using ATPE, and the two stage back extraction process previously detailed, then the product pool, formed from the pooling of the bottom phases from the two back extraction steps, will have a volume of approximately 23,000L. If it is assumed that this material can be loaded directly onto an IEX column, and maintaining the same operating conditions as before, the time required to complete the IEX chromatography cycle would now be anywhere between 5 - 6 hours, twice the time required than if Protein A were used as the primary capture step. As a result, whilst the ATPE process may have a higher productivity when compared to Protein A chromatography, as a result of the product dilution, any benefits gained by using it, may be outweighed by the increased processing times required by subsequent purification steps. This may result in the overall productivity of the process train actually decreasing.

It should also be noted that the use of three back extraction stages has draw-

backs beyond just cost and product dilution. For example the top phase volume of the final back extraction system was found to be negligible. This implies that the PEG present in top phase from the initial forward extraction system is now dispersed amongst the bottom phases of each of the back extraction systems. The purpose of the back extraction step is to recover the product mAb from the polymer rich phase into a salt rich aqueous phase, which may be taken and further purified, using for example chromatography, in order to generate a suitable final drug product. Recovery of the product in a salt rich phase is necessary since direct processing of the top phase from the forward extraction, using subsequent downstream processing operations poses several operational issues. Obviously the presence of PEG in the final drug product is highly undesirable and as such its clearance is absolutely necessary. However the presence of polymers in the process stream can cause an associated increase in fluid viscosity which can make liquid handling problematic. For example loading a viscous feed onto a packed bed chromatography column can lead to none ideal flow profiles through the column, and in extreme circumstances, viscous fingering may occur, compromising the purification performance of the chromatography step. Alternatively, the PEG may be removed using an ultrafiltration rig and membrane run in diafiltration mode. However here, the relatively high fluid viscosity may negatively impact upon membrane fluxes which in turn can significantly increase process times. The relatively large amounts of PEG present in the bottom phases of the second and particularly the third back extraction steps therefore presents an additional reason for why the three step back extraction scheme is not ideal.

Nevertheless, this multi-stage back extraction process did result in the highest yields yet seen. The increased yields were presumed to be primarily a result of the sequential dilution of the system concentration of NaCl but may also be partially attributable to the reduction in the amount of PEG present in each subsequent back extraction system. The need for this critical coupling of effects helps explain why a forward extraction system containing 2% (w/w) NaCl results in high levels of mAb precipitation, whilst the second back extraction system of the multi-stage back extraction process, which also contains a NaCl concentration of approximately 2% (w/w)

not only results in a lower level of mAb precipitation, but also provides a greater yield of mAb in the bottom phase. Based on this, it was hypothesised that the three stage back extraction process could be reduced to a two stage back extraction, whilst still maintaining the same overall mAb yield in the salt rich phase, collected from each back extraction step, by reducing the NaCl concentration in the forward extraction system. Reducing the NaCl concentration of the forward extraction system will result in a decrease in the NaCl concentration of the subsequent back extraction steps. It was hoped that such a reduction would encourage partitioning of the mAb into the bottom phase during the first back extraction stage, thereby making the third and final back extraction step unnecessary. In the original iteration of this multi-stage back extraction process, described previously, the first back operation step simply acts as a polishing step, since no mAb moves into the bottom phase during this stage, just impurities (based on the presence of a non-mAb peak in the chromatogram from the Protein A analysis of these samples). It was felt that the extra purification afforded by the back extraction step could be sacrificed since the elimination of the final back extraction step would not only decrease the associated cost of the process, but would also potentially help alleviate the product dilution and PEG concentration issues outlined previously. It was observed from the experimental array in which forward extraction systems were formed with varying concentrations of NaCl, that systems containing NaCl in concentrations of 6% (w/w) and lower resulted in high levels of mAb precipitation. Systems with NaCl concentrations of 10% (w/w) and 8% (w/w) however did not display behaviour which could be considered significantly divergent from that displayed by forward extraction systems containing NaCl at a concentration of 12% (w/w). Partition coefficients and phase ratios across these systems were comparable. Sequential back extractions were therefore performed on the top phases obtained from forward extraction systems containing 10% (w/w) and 8% (w/w) NaCl using a concentrated 30% (w/w) Na-citrate back extraction buffer, added as before, in a volume ratio, top phase to extraction buffer, of 1:1.2 to give a final system concentration of 16% (w/w) Na-citrate. The results are summarised in Table 6.9

Table 6.9: Result of Decreasing NaCl Concentration and use of a Two Stage Back Extraction Approach

Step	Partition Coefficient (K)	Phase Ratio (V_t/V_b)	mAb Yield Top Phase (Measured)	mAb Yield Bottom Phase (Measured)
Forward Extraction (10% NaCl)	175.44	0.89	156.6%	1.0%
Back Extraction I	89.96	1.67	167.0%	1.7%
Back Extraction II	5.23	0.26	73.1%	53.0%
Forward Extraction (8% NaCl)	573.35	0.89	146.5%	0.3%
Back Extraction I	67.67	1.67	158.7%	2.2%
Back Extraction II	5.49	0.24	69.0%	51.2%

It can be seen that as a result of decreasing the NaCl concentration in the forward extraction from 12% (w/w) to 10% (w/w) and 8% (w/w), in both cases the mAb yield in the bottom phase of the first back extraction increased from 1% to 2%. Whilst this is an improvement it cannot be deemed significant. A second back extraction performed in the same manner as the first upon the top phase from the first back extraction system resulted in a bottom phase mAb yield of approximately 53%, giving an overall mAb yield of around 55%. This is a slight improvement, since the mAb yield in the second back extraction step in the original iteration of the multistage back extraction process was approximately 47% (Table 6.7). Thus reducing the NaCl concentration of the forward extraction system seems to have increased the mAb yield over two back extraction steps. However a proportion of mAb still remains in the top phase following the second back extraction step. Whilst this mAb could presumably be recovered in a final back extraction step, the aim was to reduce the number of back extraction steps from three to two, for reasons previously detailed. Based on the results obtained, the bottom phase mAb yield, from the first back extraction stage, was clearly the major issue. In order to increase the mAb yield in this bottom phase, a slightly modified form of a strategy which had already been unsuccessfully tested was utilised.

A previous attempt to increase the mAb yield in the bottom phase of the back extraction system, involved lowering the citrate concentration in the back extraction system such that the operating point moved below the binodial. A concentrated shot of PEG solution was then added to the back extraction system in order to cause two phase formation. At the time, it was hoped that lowering the citrate concentration would increase the product solubility of the bottom phase of the back extraction system. At the same time, it was hoped that the addition of PEG to the system would increase the polymer concentration of the top phase. These two effects would thereby make it more thermodynamically favourable for the product mAb to move into the bottom phase. Unfortunately the results proved to be unsatisfactory. This was thought to be due to a combination of increasing PEG concentration in the bottom phase and also a decrease in the bottom phase volume which occurs since any aqueous two phase system is a close equilibrium such that changes to one phase will inevitably impact upon the other.

Accounting for this, an alternative form of this strategy was therefore proposed. It was postulated that the impact of adding PEG to the back extraction system upon the properties of the bottom phase, including its volume and PEG concentration, could be lessened by using a PEG of a higher molecular weight than that originally utilised in the forward extraction system. Thus rather than injecting a concentrated shot of PEG 1500 into the back extraction system, a shot of PEG 6000 could be used instead. It was felt that using a higher molecular weight PEG would require a smaller amount of PEG to be added in order to generate a two phase system. In the previous experiment, the back extraction system with Na-citrate concentration of 13% (w/w) required a concentrated PEG shot of approximately 14% (v/v) of the total system volume. Using PEG 6000 in place of PEG 1500, it was hoped that the size of this shot could be significantly reduced, thereby reducing the amount of PEG added to the system. This would in turn hopefully reduce the impact upon increasing PEG concentrations in the bottom phase and also have a smaller effect upon the system phase ratios.

The new back extraction strategy therefore combined the multi-stage back ex-

traction approach with the PEG injection scheme. A forward extraction system was generated based on the conditioned defined by Andrews et al., albeit with the NaCl concentration reduced from 12% (w/w) to 10% (w/w). The top phase was then recovered and contacted with a 21% (w/w) Na-citrate back extraction buffer in a volume ratio of 1:1.2 (top phase volume: back extraction buffer volume) giving a final system citrate concentration of 13% (w/w). A 6% (v/v) shot of 50% (w/w) PEG 6000 solution was then added to this first back extraction system. The system was mixed and allowed to form two phases before the top phase was recovered. The second back extraction system was formed by contacting this recovered top phase with fresh Na-citrate back extraction buffer in the same volume ratio as with the first back extraction step. Again, a 6% (v/v) shot of 50% (w/w) PEG 6000 solution was added to the second back extraction system. The bottom phases from the two back extraction systems were recovered for analysis. This experiment was also repeated, with the size of the PEG 6000 shot reduced from 6% (v/v) to 4% (v/v). The results are summarised in Table 6.10.

Table 6.10: Bottom phase mAb yields obtained using multi-stage back extraction scheme with the addition of PEG 6000 in different quantities so as to help cause two phase formation

	Bottom Phase mAb Yield (%)	
Step	6% (v/v) PEG 6000 Shot	4% (v/v) PEG 6000 Shot
Forward Extraction	1.0%	1.0%
Back Extraction I	21.9%	33.9%
Back Extraction II	47.7%	38.5%
Final Product Pool	69.6%	72.4%

The results (Table 6.10) indicate that reducing the Na-citrate concentration of the back extraction systems from 16% (w/w) to 13% (w/w), and introducing 6-4% (v/v) injections of concentrated PEG 6000 solution, have resulted in, not only an increase in the bottom phase mAb yield of the first back extraction step, but also an overall increase in the bottom phase mAb yield. The mAb obtained from the three

step back extraction process by pooling of the bottom phases from the second and third back extractions resulted in an overall yield of approximately 64%. This has increased to 72.4% using the new two stage back extraction scheme, making the yield achieved using this ATPE process at least comparable to that obtained using Protein A chromatography. Thus not only has the amount of mAb which can be recovered increased, but the number of back extraction stages has also been successfully reduced. Whilst this initially seemed to be a promising result, calculations of phase volumes still revealed a significant level of product dilution. In fact, even with the elimination of the final back extraction stage, the product pool volume was still over twice the volume of the feed. Thus whilst reducing the number of back extraction stages may make this scheme slightly more economical, the issues related to the size of equipment which would be needed to operate this scheme at a large scale, along with the problems which would be encountered by subsequent downstream processing steps, in terms of productivity, still remain.

6.6.6 Single Stage Back Extraction

Development of the back extraction process had reached a stage whereby the mAb yields obtained had reached an acceptable level. However the problem of product dilution, due to the multi-step nature of the back extraction process, remained an issue which required resolution.

The increased partitioning of mAb to the bottom phases during the multi-stage back extraction process was presumed to be caused by a combination of reduced NaCl concentrations and also a reduction in the overall system polymer concentration. Whereas previous multi-stage back extraction schemes had concentrated on lowering the NaCl concentration, it was decided that an interesting avenue of investigation would be to observe the effect of reducing the polymer concentration of the back extraction system. The lowering of the NaCl concentration had previously been accomplished by firstly lowering the salt concentration in the first forward extraction system, after which the non-preferential partitioning of NaCl ions was exploited in each subsequent back extraction system, allowing a gradual reduction in the con-

centration of Cl- ions in the ATPE systems. The same approach could not be used to reduce the polymer concentration of the back extraction systems since firstly the PEG concentration in the forward extraction system seemed to be resulting in the formation of two phase systems with favourable partitioning characteristics. That is, there seemed to be a high yield of mAb in the top phase of forward extraction systems employing PEG concentrations of 15% (w/w). Also the reduction in the PEG concentration as a result of sequential back extraction systems was not as dramatic as that seen for NaCl because in a back extraction two phase system, PEG will not partition equally throughout both top and bottom phases (as is the case with NaCl). Instead, whilst a small amount may be present in the bottom phase, the majority of PEG will be present in the less dense top phase and as such will be carried over into the next back extraction stage.

In order to reduce the polymer concentration of the back extraction system without affecting that of the forward extraction, a more extreme approach was adopted. The forward extraction was performed as before based upon the conditions defined by Andrews et al., with component concentrations of 15% (w/w/) PEG 1500, 14% (w/w) Phosphate and 12% (w/w) NaCl. The top phase from this forward extraction was then recovered, but rather than being immediately subjected to the back extraction process, it was instead diluted with 25% (v/v) RO water. Thus for example 15mL of top phase from the forward extraction was diluted with 5mL of RO water. After sufficient mixing, this diluted top phase was then contacted with an 18% (w/w) Na-Phosphate back extraction solution with a pH of 3.2, in order to form the two phase back extraction system. A phosphate rather than a Na-citrate solution was used in order to ensure that the reduction in the polymer concentration did not result in the operating point for the back extraction system moving below the binodial curve of the phase diagram. Previous experiments had shown that the binodial curve shifted to high polymer and salt concentrations upon moving to the Na-citrate back extraction buffer.

The reduction of the back extraction process to a single step had the desired effect upon the product concentration. The bottom phase of this back extraction

process had a volume which was only 1.2 times the volume of the original cell culture supernatant feed to the forward extraction system. Whilst this is still not ideal, it is a significant improvement over the dilution factor of 2 obtained using the two step back extraction approach. Unfortunately the yield obtained using this single step approach was only 49%. In addition to this the phase ratio was approximately 0.21 and there was a significant amount of precipitation present, implying that a relatively large proportion of mAb had been lost in the precipitate, even after accounting for the possible overestimation of the concentration of mAb in the cell culture supernatant feed. Thus whilst the issue of product dilution has been addressed to a certain extent, the process yield is now too far beyond that which would be considered acceptable for large scale manufacturing.

6.6.7 Alternative ATPE System

Based on the results from this protracted development of the back extraction process, an obvious observation must be made, and that is the conspicuous divergence of the behaviour of this particular ATPE process from that observed by Andrews et al., when originally developing the system. In light of this, an effort was made to ascertain whether the level of divergence observed was isolated to only this particular ATPE system, or whether it also applied to other ATPE systems. Azevedo et al., developed an ATPE system with a composition of 12% PEG, 10% Phosphate and 15% NaCl.[111] This same ATPE system was applied to the feed used in this study in order to determine if the behaviour was comparable. In the original study, a CHO cell culture supernatant, containing mAb at a concentration of approximately 0.5g/L, and a HCP content of approximately 5 million ppm was used. In this system the mAb was found to preferentially partition to the top phase with a K value of 36.4 and a top phase mAb yield of 88.4%. No interfacial precipitate formed. However when the same system was used for the feed in this study, even though a comparable partition coefficient was observed (K=24), the yield of mAb in the top phase was significantly lower at approximately 18%. Furthermore the remaining mAb was not present in the bottom phase which had a mAb yield of only 1%. Instead, the mixing

of the system components with the feed, resulted in the formation of a thick interfacial precipitate, which based on a mass balance, must contain the remaining mAb. This once again represents a significant divergence from the expected performance of the ATPE system developed by Azevedo et al.

6.6.8 Summary of ATPE Development

It is clear from the results of the development of the back extraction step of the ATPE system developed by Andrews et al., that there seems to be a thermodynamic hindrance to the partitioning of mAb to the bottom phase during the back extraction stage of the ATPE process. Numerous methods have been tested, involving changing the composition of both the forward and backwards extraction systems in order to manipulate the position of the operating point relative to the binodial curve, so as to improve the yield in the salt rich bottom phase during the back extraction. From the results presented, it is evident that in order to obtain any appreciable recovery of mAb in a salt enriched phase, it will be necessary to employ multiple back extraction steps. Taking such an approach however leads to a significant amount of product dilution, an outcome which is not desirable for a primary capture unit operation. An alternative approach would be to simply eliminate the back extraction step, and process the top phase from the forward extraction. Product recovery in the top phase following forward extraction is high. The forward extraction also results in an approximate 2-fold concentration of the product. Processing of the top phase does however have several drawbacks. Firstly the purification factor achieved by the step may be compromised as a result of elimination of the back extraction steps. Also, the high PEG content of this top phase may lead to its own set of processing challenges. The most suitable method for product recovery for further processing will be highly dependent upon the nature of the step following ATPE. If the step can handle feeds with high viscosity, then recovery and processing of the top phase may be a viable alternative. Also if the following steps are of a sufficient selectivity then the loss of resolving power during the ATPE process would also not be a major issue. If however direct loading of the top phase from the forward (or indeed first backward) extraction

system onto the subsequent process in the process train is not a feasible option, it will be necessary to perform the multi-stage back extraction process, in order to recover the product in a salt enriched phase. A ultrafiltration/diafiltration step may then be necessary in order to reduce the process volume. The integration of ATPE into the platform process is further detailed in the following chapter.

Whilst the primary aim of this development work was to generate an ATPE process which could be integrated into the platform process, the results also provide some insight as to the characteristics of ATPE processes in general. Over the course of these development experiments, two separate ATPE systems were tested, one developed by Andrews et al.[110] and the other by Azevedo et al.[111]. Neither of these ATPE systems behaved in the manner in which they were expected, with the partitioning of the product antibody deviating significantly from that described by the original developers of the systems. Given the nature of mechanisms of separation employed by ATPE systems, some variation in process performance would be expected when moving from one feed material to another, with perhaps deviations in the process yields and purification factors observed. However the magnitude of the deviations from expected performance in the case of these ATPE systems could be taken as indicators of either a specific issue associated with this particular product or as a problem with the general robustness of ATPE processes.

The issues of product precipitation and also low partitioning into the salt enrich bottom phase of the back extraction systems could be a sign that the product antibody in this case is fairly hydrophobic and therefore has a high affinity for the polymer rich top phase, and only a low affinity for the ionic bottom phase. This would explain the high partition coefficients during the back extraction steps, during which the product is meant to move into the bottom phase and partition coefficients are supposed to be <1.

Alternatively, the deviation in performance may simply be an inherent characteristic of all ATPE systems. Component partitioning during an ATPE process is highly dependent upon a wide range of different and interacting parameters, including not only the characteristics of the ATPE system itself, but also that of the feed

material such as its composition and concentration. It is therefore not inconceivable that such a system would be highly sensitive to slight variations in the process feed, and that therefore a "generic" ATPE process is not a realistic concept. This lack of robustness may be an issue when it comes to process validation. ATPE is being used as a primary capture step, where it will be exposed to feed material which is inherently variable. If the performance of the ATPE process changes dramatically with slight changes in product or impurity concentrations, then this increases the burden on downstream process development, which must not only develop ATPE processes which provide satisfactory performance, but must also be able to handle deviations in the feed properties caused by upstream variability. The aim of process validation is to ensure that the process behaves in a desired manner, maintaining critical product attributes by controlling the operating parameters within defined ranges. The sensitivity of the ATPE process of changes in the feed characteristics means that these defined operating ranges must be very tight, in order to ensure consistent and predictable performance. Due to the batch to batch variability often seen in upstream operations, this level of control may not necessarily be possible. For example it may only be possible to ensure the performance of an ATPE process if the feed product concentration is between 0.9 and 1.1 g/L. However if the upstream process results in cell culture titres of anywhere between 0.8 to 1.2 g/L, then the ATPE process cannot be validated for this purification. The situation is further exacerbated by the fact that the precise mechanisms of separation utilised by ATPE are only partially understood. As a result, it is difficult to determine precisely, what the critical process parameters which need to be controlled, actually are. Even if the critical process parameters could be identified, the nature of the ATPE process means that control of said parameters may not necessarily be straightforward. An example is the system pH, which in the case of the forward extraction step of the ATPE process evaluated in this study, is dependent upon the ratio of salts added to form the two phase system. Because the system pH is determined in this manner, rather than for example, through the addition of a pre-prepared buffer which can be checked against set acceptance criteria prior to use, any deviations in system pH are difficult to correct. Addition of a titrant

is not feasible since this will affect the composition of the system which in turn may impact upon the partitioning behaviour of the product. This means that very tight limits must be placed upon acceptable masses of each phase forming salt which can be added to form the ATPE forward extraction system, to ensure that the correct pH is reached each time the process is run.This also makes no account of variability in the feed pH. These problems with control further increase the difficulty of validating the ATPE process.

As stated previously, the difficulty of process validation places an increased burden on downstream process development. This is worsened by the fact that based on the deviations in process performance which have been observed in this study, ATPE systems would need to be developed almost from scratch for each new product, with the use of high throughput screening approaches for development and optimisation. It is clear from the work performed in this study that a rationalised stepwise approach to the development of an ATPE process, is not the most efficient strategy.

6.7 HPTFF Development

Charged ultrafiltration membranes are currently not commercially available. As a result, in order to evaluate the use of HPTFF using a charged membrane, the membrane itself had to firstly be synthesised. A recommended procedure for adding charge to membranes has been previously detailed[112]. The method essentially involves exposing the membrane to a reactant solution containing a charged ligand. The reaction then causes this ligand to be immobilised onto the membrane surface imbuing the membrane with a charge, the polarity of which is dependent upon the ligand used.

In this instance, a 300kD composite regenerated cellulose (CRC) ultrafiltration membrane was reacted with a solution containing 3-bromopropyl trimethyl ammonium bromide, a compound which carries a positive charged under pH conditions typically used for protein separation (pH 4 -8). This alkyl halide reacts with the OH groups of the primary alcohols in the CRC matrix forming covalent ether bonds thereby immobilising the ligand to the membrane surface. Figure 6.9 shows the base

catalysed reaction and the formation of the ether bond.

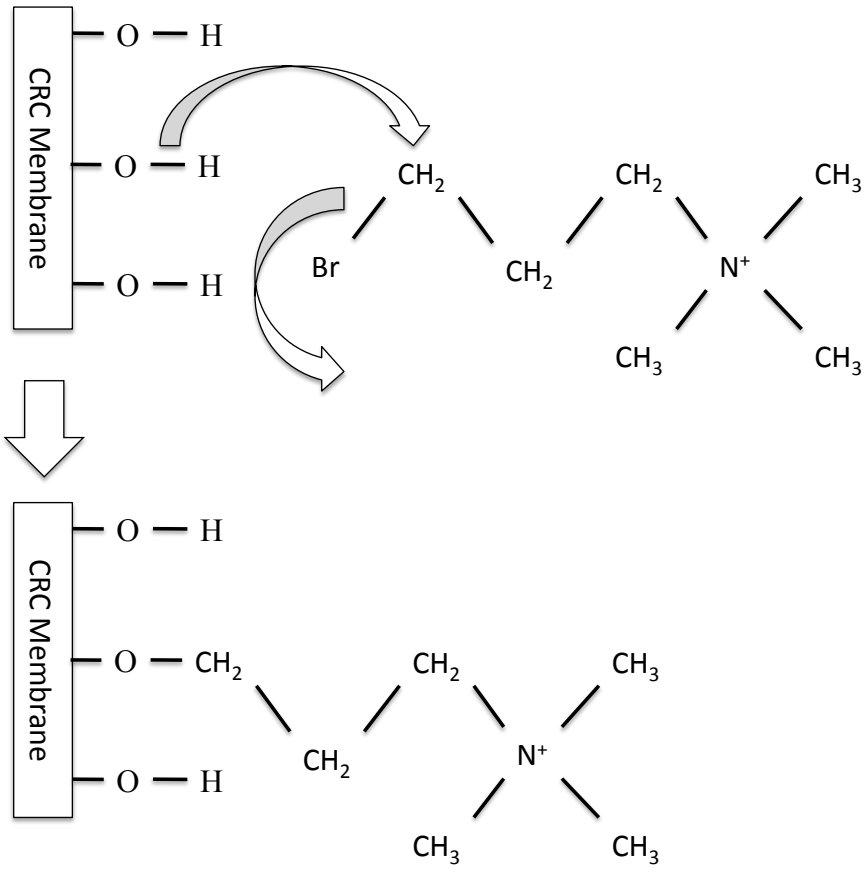


Figure 6.9: Schematic showing formation of covalent ether bonds between 3-bromopropyl trimethyl ammonium bromide and OH groups of primary alcohols in the cellulose matrix, leading to immobilisation of the charged ligand to the surface of the CRC membrane

6.7.1 Choice of membrane MWCO

Use of HPTFF as a purification step requires a decision to made as to whether to collect the product in the permeate or to keep it in the retentate. This decision will then help to determine the exact MWCO of the membrane to be used. In this study, the aim was to purify a monoclonal antibody, which will typically be one of, if not the largest protein in a mammalian cell culture supernatant. For this reason it makes more sense to try and keep the mAb in the retentate whilst allowing other

smaller components to pass through into the permeate. The issue then becomes what membrane MWCO to use. A excessively small pore size may maximise the mAb yield in the retentate, but will also limit the flux as well as the purification factor achievable due to the high retention of impurities. Alternatively an excessively large MWCO might allow high purification due to increased transmission of impurities, but it also might lead to low product yields, as more mAb is allowed to pass into the permeate. Strict rules of thumb regarding the membrane MWCO which should be used based on protein molecular weights should generally be avoided as the hydrodynamic radius of a protein, which ultimately determines its size, is highly dependent upon the ionic strength and pH of the system and will therefore be specific to particular sets of operating parameters.

Commercially available CRC membranes come in a number of different, discreet MWCOs; 5kD, 10kD, 30kD, 100kD, 300kD and 1,000kD etc. From this range the 5kD, 10kD and 30kD can likely be eliminated as these MWCO are most likely too small, whilst the 1,000kD membrane may be eliminated for being too large. This leaves the choice between the 100kD and the 300kD membrane. In this study, the decision was made to use a 300kD membrane for a number of reasons. Firstly it has been documented that adding charge to the cellulose membrane though immobilisation of the charged ligand results in a corresponding decrease in effective membrane pore size[112]. The cut-off of 100kD is smaller than the product mAb, which would in turn ensure a relatively high yield. However as discussed it may also limit the throughput and purification power of the HPTFF step. Adding charge to the membrane would reduce this cut-off further, thereby exacerbating the situation. Using the 100kD membrane would also not allow the advantages of membrane charge to be fully exploited. The use of a charged membrane allows components to be retained based not only on their size but also their charge. Thus if there is a impurity present in the feed with the same molecular weight as the IgG but a significantly lower pI, use of a 100kD membrane would not allow these components to be separated, as both would be retained by the membrane.

If however the ultrafiltration were to be performed at a pH close to the pI of the

impurity, using a positively charged 300kD membrane, separation should be theoretically possible. The membrane MWCO is larger than both components and if electrostatic interactions are ignored then both components should be able to pass relatively unhindered into the permeate. However the use of a positively charged membrane will mean that the IgG may actually be retained by the membrane through electrostatic repulsion forces, whilst the neutral impurity will pass into the permeate thereby affected purification of the product antibody.

For these reasons the decision was made to use a 300kD membrane which is in line with the suggestions made by the originators of the technology.[115]

6.7.2 Uncharged Membrane Characterisation

The characteristics of the ultrafiltration membrane in its uncharged form were initially evaluated in order to provide a baseline against which the performance of the charged membrane could be compared. These evaluations essentially involved challenging the membrane with a two-component mixture containing a polyclonal IgG and BSA, and determining the sieving properties of the membrane under a range of different pH and ionic strength conditions. These evaluations were all performed with a 300kD Ultracel Pellicon XL50 membrane cassette installed in the custom built HPTFF system (based on the use of an AKTAcrossflow system) described previously. Feed was delivered to the membrane at a constant flow rate of 323 LMH. The retentate pressure was adjusted to 0.69 bar using the manual pressure valve and the co-current flow rate was adjusted to obtain equal transmembrane pressure across the membrane cassette. The system was operated in total recycle mode. Flux through the membrane was controlled at 50, 100 and 150LMH using the feedback controls of the AKTAcrossflow system.

Low Ionic Strength, Low pH

The performance of the membrane was initially evaluated with the feed formed by dissolving the polyclonal IgG and BSA in 10mM Na-acetate at pH 4.0. Under these conditions the polyclonal IgG was expected to carry a strong net positive charge, as the pH is significantly lower than the average pI (approximately 8-9) of this component.

The BSA on the other hand should carry only a very small net positive charge since the pH is so close to its pI (approximately 4.6). The results of these experiments are summarized in Table 6.11.

Table 6.11: Sieving coefficient and selectivity of uncharged membrane in 10mM Na-Acetate pH 4.0

Flux (LMH)	BSA Sieving Coefficient (S1)	IgG Sieving Coefficient (S2)	Membrane Selectivity (Ψ)
50	0.63	0.30	2.08
100	0.66	0.27	2.46
150	0.59	0.11	5.45

The sieving results of the uncharged 300kD membrane at fluxes of 50 and 100LMH were fairly unremarkable considering the conditions, which were used. Both BSA and the polyclonal IgG have molecular weights below the membrane MWCO and as a result both experienced significant levels of transmission through the membrane, as evidenced by the relatively high sieving coefficients. The sieving coefficient of the BSA was predictably larger than that of the IgG due to the smaller size of BSA however under flux conditions of 50 and 100LMH, the selectivity of the membrane is fairly comparable.

An interesting result was obtained when the flux through the membrane was increased to 150 LMH. At this flux, a significant increase in the selectivity of the uncharged membrane was observed. This increased selectivity is almost entirely attributable to a decrease in the sieving of IgG through the membrane as the sieving of BSA was relatively unaffected by the increase in flux. It was thought that the increased level of IgG retention by the uncharged membrane and resultant increase in membrane selectivity was caused by electrostatic charge repulsion between positively charged IgG molecules. The feed rate was maintained at 323 LMH in all of the experiments performed. Maintaining the feed flow rate and increasing the flux from 50LMH to 150LMH would have also resulted in a concomitant decrease in the cross-flow rate across the membrane surface on the retentate side. A reduction in

tangential flow would have in turn lead to an increase in concentration polarization of molecules at the membrane surface. In tangential flow filtration operations, the level of concentration polarisation at the membrane surface, during operation, must be carefully optimised. Increasing the level of polarisation of a component can lead to an increase in the membrane flux of that particular component, as the high concentration of molecules at the membrane surface will lead to more passing through into the filtrate. However excessive polarisation can lead to the formation of an impermeable gel layer and membrane fouling. The results for BSA sieving, shown in Table 6.11, serve to illustrate this careful balance. At a membrane flux of 50 LMH, BSA has a sieving coefficient of 0.63. As the flux is increased to 100 LMH, as stated the cross flow rate decreases and concentration polarisation at the membrane surface increases. The increase in local concentration of BSA at the membrane surface results in an increased in the flux of BSA causing the sieving coefficient to increase by approximately 5% to 0.66. As the solvent flux is increased further to 150 LMH, the level of concentration polarisation increases even further to a point whereby fouling of the membrane starts to cause a decrease in the BSA solute flux, with the associated sieving coefficient dropping by approximately 11% to 0.59.

Whilst there is some change in the sieving coefficient of BSA across the membrane fluxes tested, the differences here are small compared to those observed with the sieving coefficient of the IgG across this flux range. As stated the BSA sieving coefficient varied only by 5-11%, whereas the IgG sieving coefficient was seen to drop by over 60% at the highest flux. Furthermore, the sieving coefficient of IgG was not seen to reach a maximum at the intermediate solvent flux of 100 LMH.

The reason for these deviations is most likely due to the differences in the charge characteristics of IgG and BSA. Under the pH conditions of this experiment (pH 4.0) the BSA molecules would have been either net charge neutral or possibly very slightly positively charged. The IgG molecules meanwhile would have been highly positively charged. Increasing the level of concentration polarisation through elevation of the solvent flux would therefore have caused not only the build-up of a layer of neutral BSA molecules but also a layer of highly positively charged IgG molecules at the

membrane surface. This positively charged layer would have in turn repelled other IgG molecules from the membrane surface leading to the observed decrease in IgG sieving and the increase in membrane selectivity. BSA sieving meanwhile is unaffected by these charge effects since these molecules are neutrally charged.

The sieving of BSA was seen to increase as the flux was increased from 50 LMH to 100 LMH due to concentration polarisation effects. The sieving of IgG on the other hand decreased by approximately 10% from 0.30 to 0.27 over the same interval as presumably the increase in IgG flux due to concentration polarization is outweighed by the electrostatic repulsion imposed by the positively charged layer of molecules. As the solvent flux increased further to 150LMH, the level of concentration polarization increases and thus so does the size of the positive charge presented by the layer of molecules on the membrane surface. At this point the electrostatic repulsion of IgG from the pores of the membrane greatly outweighs any increased transmission of IgG due to higher concentrations at the surface leading to the observed drop of 60% in the IgG sieving coefficient.

High ionic strength, high pH

In order to further explore the effects of molecular charge on membrane sieving properties, the uncharged membrane was tested under conditions of increased ionic strength and pH. Specifically the IgG and BSA were suspended in 150mM Na-Phosphate pH 7.0 buffer. Under these conditions the BSA molecules carry a net negative charge, whilst the IgG molecules, as at pH 4.0, will carry, albeit a smaller, net positive charge. Table 6.12 summarises the performance of the uncharged membrane under these conditions.

Increasing the operating pH and ionic strength of the feed resulted in a decrease in the selectivity of the uncharged membrane. Based upon the sieving coefficients at corresponding fluxes of 50 and 100 LMH, this reduction in selectivity is almost entirely attributable to a decrease in BSA sieving. Comparing the results shown in Tables 6.11 and 6.12, it can be seen that the sieving coefficients of the IgG seem to be relatively unaffected by the changes in the physicochemical environment. However

Table 6.12: Sieving coefficients and selectivity of uncharged membrane in 150mM Na-Phosphate pH 7.0

Flux (LMH)	BSA Sieving Coefficient (S_1)	IgG Sieving Coefficient (S_2)	Membrane Selectivity (Ψ)
50	0.53	0.29	1.83
100	0.48	0.29	1.62

there has been a significant decrease in BSA sieving as a result of the increases in ionic strength and pH.

The increased retention of BSA by the membrane is most likely due to the increased charged density of the BSA molecules. Under the low pH conditions, the BSA molecules retained a net neutral charge. However under neutral pH conditions, BSA molecules carry a net negative charge. The increase in electrostatic charge density will lead to a concomitant increase in the hydrodynamic radius of the BSA molecules. This increase in molecular size therefore leads to an increase in BSA retention, which results in the observed decrease in membrane selectivity. The decrease in BSA sieving may also be slightly attributed to the same electrostatic repulsion effects observed with IgG under low pH and ionic strength conditions. The size of such repulsive forces would most likely not have been as large as those experienced by IgG under the low pH and ionic strength conditions, as firstly the operating pH is not as far from the pI of the BSA and also the relatively high Na-Phosphate concentration of 150mM would have served to disrupt some of the electrostatic repulsion forces which may have been in effect. Nevertheless such interactions cannot be completely discounted and would have played a role in increasing the retention of BSA under this new set of conditions.

The sieving coefficient of IgG at a flux of 50 LMH is comparable to that observed under the low ionic strength and pH conditions (0.30 at pH 4.0 compared to 0.29 at pH 7.0), whilst the sieving coefficient at a flux of 100LMH is slightly higher at the neutral pH.

However, unlike as was observed at the low pH conditions, no decrease in the

sieving coefficient occurs at the neutral pH, as the flux is increased from 50 LMH to 100 LMH. This is likely due to the reduced electrostatic interactions between IgG molecules. Increasing the pH of the system will have resulted in a decrease in the size of the net positive charge carried by the IgG molecules. Combined with the increased ionic strength, the same electrostatic repulsion effects, which decreased the sieving coefficient of IgG under the low pH conditions as the flux was increased, are now significantly reduced. As a result the sieving coefficients of IgG at 50 LMH and 100 LMH are approximately equal at these high ionic strength and pH conditions.

The sieving coefficients of IgG and BSA at a flux of 150 LMH could not be determined at the increased ionic strength and pH conditions, since the transmembrane pressure required to achieve this flux was beyond that which the Pellicon XL 50 cassette may be operated, indicating that an excessive level of membrane fouling had occurred, most likely as a result of the decrease in the cross-flow rate. As discussed previously, increasing the flux whilst maintaining the feed flow rate results in a decrease in cross-flow, which in extreme cases, causes excessive concentration polarization and the formation of an impermeable gel layer across the membrane surface.

Interestingly this level of membrane fouling did not occur at the lower ionic strength and pH conditions which suggests that the increased level of concentration polarisation must be attributable to the relative changes in the electrostatic interactions between IgG and BSA molecules.

As was described previously, under conditions of low ionic strength and pH, the build-up of highly positively charged IgG molecules at the membrane surface resulted in electrostatic repulsion of other IgG molecules, leading to a concomitant decrease in IgG sieving. Increasing the ionic strength and pH would have served to diminish these electrostatic forces. Therefore, based on these results, it seems that the electrostatic interactions which caused a decrease in the sieving coefficient of IgG, also helped to reduce the level of concentration polarisation and hence membrane fouling which occurred. The absence of said interactions at the higher pH, leads to more rapid formation of the impermeable gel layer and increased levels of membrane fouling, making it impossible to achieve a flux of 150 LMH.

By this rationale, the neutrally charged BSA molecules should also have caused fouling of the membrane at the low pH and ionic strength conditions in the same way that the IgG is proposed to have behaved at the higher ionic strength and pH conditions. However since a flux of 150 LMH was achievable at the low pH conditions, it must be assumed that IgG is the bigger contributor to membrane fouling. This is mostly likely due to a combination of two factors. Firstly IgG was present in the feed at a higher mass concentration than BSA, although it should be noted that the number of molecules of IgG and BSA in the feed would have been comparable. The second factor is the relative size of these molecules. A BSA molecule is about half the size of an IgG molecule and therefore, ignoring charge effects, the level of concentration polarisation of BSA at the membrane surface would have been less than IgG, simply as a result of higher transmission of BSA through the membrane. Indeed the sieving coefficient of BSA is twice that of IgG at the low ionic strength and pH conditions.

6.7.3 Purification Factor and Yield of Ultrafiltration with Uncharged Membrane

The result of these membrane evaluations indicate that even without the use of a charged membrane, interesting electrostatic interactions still play a role in protein ultrafiltration, based on the physicochemical environment of the components which are to be separated. By operating at a pH far from the pI of the component to be retained (thereby causing molecules to carry a net charge), and close to that of the component(s) to be passed into the permeate, it was possible to greatly enhance the retention of the highly charged component through combined effects of concentration polarisation and charge repulsion. Furthermore these effects were seen to help to reduce the level of membrane fouling which occurred.

However, whilst the choice of ionic strength and pH conditions may be used to enhance the separation of BSA and a polyclonal IgG, the results show that the level of selectivity, which was achieved through manipulation of these conditions, was still not sufficient so as to provide satisfactory separation of theses two components.

The optimisation of any ultrafiltration process will normally involve the development of operating conditions so as to attain the optimum trade-off between process yield and purification factor. A technique for the optimisation of ultrafiltration processes based on the use of optimisation diagrams has been previously developed.[116] This technique relies on the use of relationships between the process yield and purification factor of an ultrafiltration process under conditions of constant throughput and constant membrane selectivity.

The exact relationships between the purification factor and yield are dependent upon the location of the product. In this study, the product IgG is kept in the retentate, in which case, the purification factor (PF) under conditions of constant throughput is given by Equation 6.1.

$$PF = e^{N\Delta S} \tag{6.1}$$

It can be seen that with the product in the retentate, the purification factor is independent of the process yield, and instead is determined by the difference between the sieving coefficients of the less retained and more retained components (ΔS) and also the number of diavolumes of buffer used for the diafiltration (N).

The difference in sieving coefficients parameter (ΔS) will always have a positive value as it is calculated by taking the sieving coefficient of the more retained component from that of the less retained component. In the case of the separation of IgG and BSA, ΔS may be expressed in terms of the sieving coefficients of BSA (S_{BSA}) and IgG (S_{IgG}) as shown in Equation 6.2.

$$\Delta S = S_{BSA} - S_{IgG} \tag{6.2}$$

The number of diavolumes (N) may be broken down into system specific operating parameters of solvent flux (J), membrane area (A), process time (t) and retentate volume (V), as shown in Equation 6.3

$$N = \frac{JAt}{V} \tag{6.3}$$

The relationship between purification factor and yield under conditions of constant membrane selectivity is shown in Equation 6.4.

$$PF = Y^{1-\Psi} \tag{6.4}$$

Plotting these relationships on logarithmic axes of Yield (y-axis) against Purification Factor (x-axis) results in a series of curves and lines describing the performance of the ultrafiltration process. The only parameters required to generate these curves are the membrane selectivity, for the particular separation of interest and the number of diavolumes to be used. The intersection of these lines then represent possible operating points with the ideal operating window being located towards the top right of the plot area (i.e. high yield and purification factors).

With the product in the retentate the diagram is simplified since the process yield is independent of the purification factor at constant process throughput. This relationship (Equation 6.1) may therefore be simply represented by a vertical line on the Yield vs Purification Factor chart, the location of which will be dependent upon the membrane flux (J), membrane area (A), process time (t) and retentate volume (V) and the difference between the sieving coefficients of the feed components (ΔS). Whilst the sieving coefficients will be dependent upon the characteristics of the membrane, the remaining parameters are all operator defined and therefore NΔS may in theory take any desired value.

Plotting of the second relationship between Purification Factor and Yield at constant selectivity, shown in Equation 6.4, onto the Yield vs Purification factor axis results in an exponential curve, the shape and location of which is dependent upon the membrane selectivity. Figure 6.10 shows the results of plotting this relationship using the selectivity values obtained for the uncharged membrane in the separation of IgG and BSA.

Figure 6.10 illustrates the trade-offs between process yield and purification factor for an ultrafiltration process. As stated previously intersecting points between the vertical lines of constant throughput and the exponential constant selectivity lines

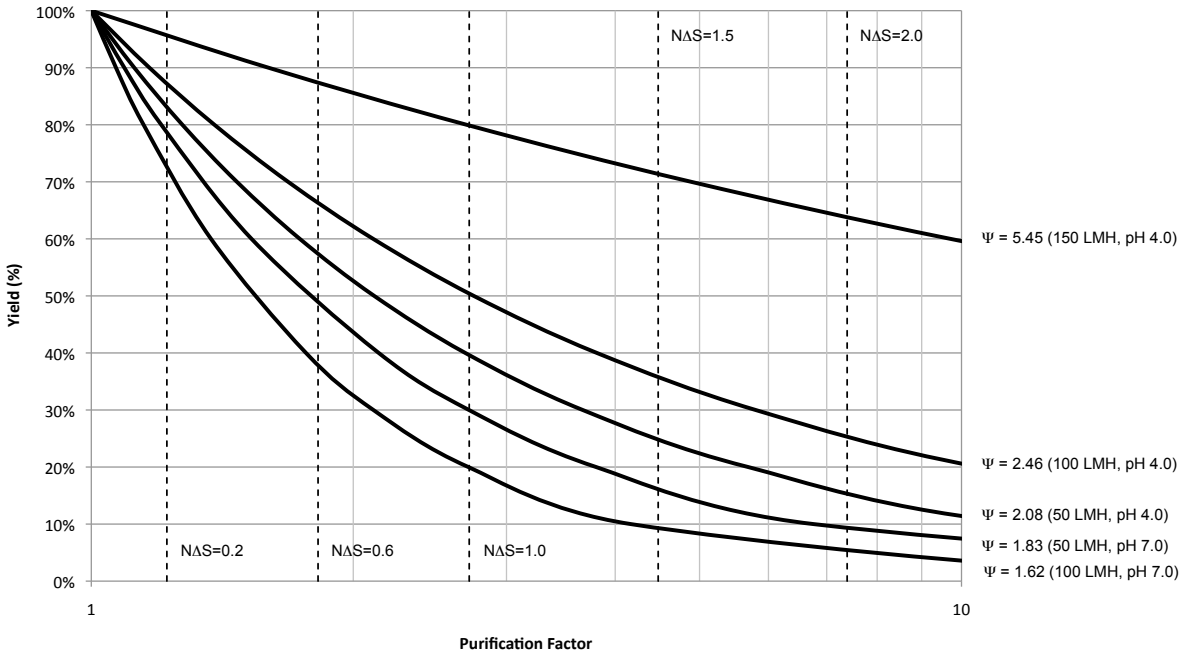


Figure 6.10: Optimisation Diagram showing relationship between yield and purification factor for the Uncharged 300kD Ultracel membrane, at different fluxes and hence selectivities for the separation of BSA and IgG

represent possible corresponding values of yield and purification factor for the ultrafiltration system, based upon the membrane selectivity and throughput.

The vertical dotted lines in Figure 6.10 represent the relationships between yield and purification factor (as described by Equation 6.1) at a constant throughput with NΔS values of 0.2, 0.6, 1.0, 1.5 and 2.0. As stated previously, with the product in the retentate, NΔS may be made to take any value through appropriate design of the ultrafiltration system. As a result corresponding yields and purification factors from this ultrafiltration system may take any value along the solid curves in Figure 6.10.

Figure 6.10 therefore shows that the purification factor of this ultrafiltration process may be increased at the expense of a loss in yield by increasing the value of NΔS, moving from left to right along the solid curves. Assuming that the membrane selectivity remains constant, increasing the value of NΔS would essentially involve increasing the number of diavolumes used during the ultrafiltration. As this is increased, the amount of impurities which pass into the permeate will increase leading to a rise in product purity in the retentate, however a concomitant increase in product transmission through the membrane will also occur leading to a decrease in product yield.

For the separation of the IgG and BSA mixture using the uncharged membrane, it can be seen that the purification factors, which may be achieved at acceptable levels of yield, are very low. In order to obtain an IgG purification factor of 10, under the conditions tested, the corresponding process yields would range predominantly between 5% and 20%. Only operation at 150 LMH under the low ionic strength and pH conditions would allow a yield of 60%, which would still not be considered an acceptable level. As a result, based on its observed performance, the uncharged membrane is clearly not suitable for performing this particular separation of IgG and BSA.

6.7.4 Normalised Water Permeability (NWP)

The same Ultracel 300kD Pellicon XL50 membrane cassette was used for all of the membrane studies which were performed. As a result it was important to be able to

quantify any changes to the condition of the membrane over the course of these studies, in order to account for their impact upon the performance of the HPTFF process. A quantity useful for monitoring the condition of a membrane is the normalised water permeability. This is essentially a measure of the relationship between the water flux through the membrane and the transmembrane pressure (TMP). The NWP of a membrane is normally expressed in units of LMH/ psi and will usually be seen to decrease as membrane fouling increases.

By monitoring the NWP before and after each experiment which was performed, it was possible to determine whether any membrane fouling had occurred and also if the fouling and resulted in a significant and irrecoverable loss of membrane permeability. Figure 6.11 plots the changes in NWP over the course of the first protein separation experiments, in which the membrane was used in its uncharged form and also the changes in the NWP as a result of adding charge to the membrane.

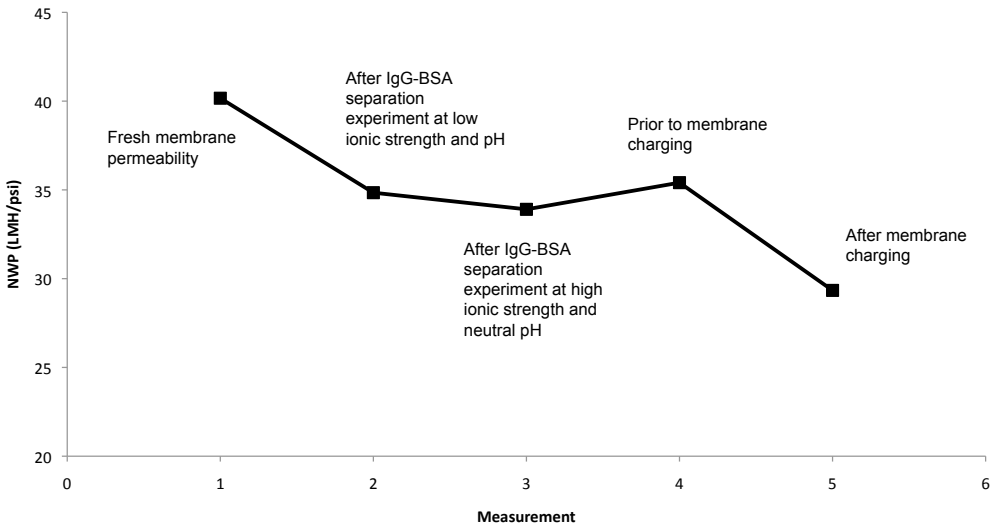


Figure 6.11: Change in the Normalised Water Permeability (NWP) of the membrane over the course of the initial studies performed

The first measurement of the NWP was performed on the fresh membrane prior to it being used for any protein separations. The new membrane was flushed with MilliQ water to remove the suppliers storage solution before measurements of the NWP were

taken.

The second measurement of NWP was performed after the first protein separation experiment in which the sieving of IgG and BSA at low ionic strength and pH conditions was tested. This measurement was performed after the membrane had been cleaned with 0.5M NaOH and thoroughly rinsed with MilliQ water. A drop of approximately 13.2% in the NWP was observed after this first experiment. This result was not completely unexpected, as ultrafiltration membranes commonly exhibit some loss in permeability following their first use. Indeed membrane permeability may drop as much as 20% in such circumstances. The loss of 13.2% in NWP was therefore well within that which would commonly be accepted.

The third NWP measurement was performed following the second protein separation experiment in which the sieving of IgG and BSA at high ionic strength and neutral pH conditions was tested. This measurement was again performed after the membrane had been cleaned with 0.5M NaOH and then thoroughly rinsed with MilliQ water. A slight decrease in the NWP, of approximately 2.7%, was observed, which is significantly less than the drop observed after the first protein separation experiment. Furthermore an additional wash step with 0.5M NaOH (Datapoint 4) allowed the NWP to be returned to that measured after the first protein separation experiment. Thus after the initial drop of 12.5%, the NWP seems to have reached a stable level (approximately 35 LMH/psi). Datapoint 5 represents the NWP measured after charging of the membrane had been completed. As can be seen charging of the membrane resulted in a significant loss of membrane permeability (approximately 25% compared to that of the fresh membrane). This is something which had been reported in the patent by van Reis[112], and is probably due to the presence of the charged ligand reducing the effective pore size of the membrane.

6.7.5 Charged Membrane Characterisation

The charged membrane was initially tested, as with the uncharged membrane, under conditions of low ionic strength and pH. The feed used was once again IgG and BSA suspended in a 10mM Na-Acetate pH 4.0 buffer. Table 3 summarises the results from

this set of experiments.

Table 6.13: Sieving coefficient and selectivity of charged membrane in 10mM Na-Acetate pH 4.0

Flux (LMH)	BSA Sieving Coefficient (S_1)	IgG Sieving Coefficient (S_2)	Membrane Selectivity (Ψ)
50	0.28	0.02	15.83
100	0.36	0.01	35.80
150	0.34	0.00	68.97

Comparing the results shown in Table 6.13 to those in Table 6.11, in can be seen that there has been a dramatic increase in the membrane selectivity. This increase is almost entirely attributable to the large decrease in IgG sieving. Whilst the results of the NWP tests showed that the charging of the membrane did result in a decrease in membrane permeability as a result of the charging reaction, this is not sufficient to explain the large drop in IgG sieving which is seen. Indeed the sieving of BSA has also decreased, but nowhere near to the same degree as that of the IgG. The sieving coefficient of BSA has dropped from approximately 0.6 to around 0.35. Comparing the results in Table 1 and 3 this represents an average decrease, across the 3 fluxes, of approximately 50%. This decrease in sieving is most likely due to the reduction in membrane permeability as a result of the charge addition. However the sieving of IgG has dropped from around 0.3 (0.1 at a flux of 150 LMH) to values of between 0.02 and 0.005. This represents an average decrease of over 95% across the fluxes tested. The increased level of IgG retention must be due to the charge repulsion caused by the positively charged membrane. At a pH of 4.0, the IgG carried a very strong net positive charge. As a result IgG will be strongly repelled by the positively charged membrane. BSA on the other hand will be net neutral and as a result can move through the membrane relatively unhindered.

Figure 6.12 shows a comparison between the sieving of IgG and BSA by the charged and uncharged membrane.

From Figure 6.12 the disproportionate reduction in IgG sieving compared to that

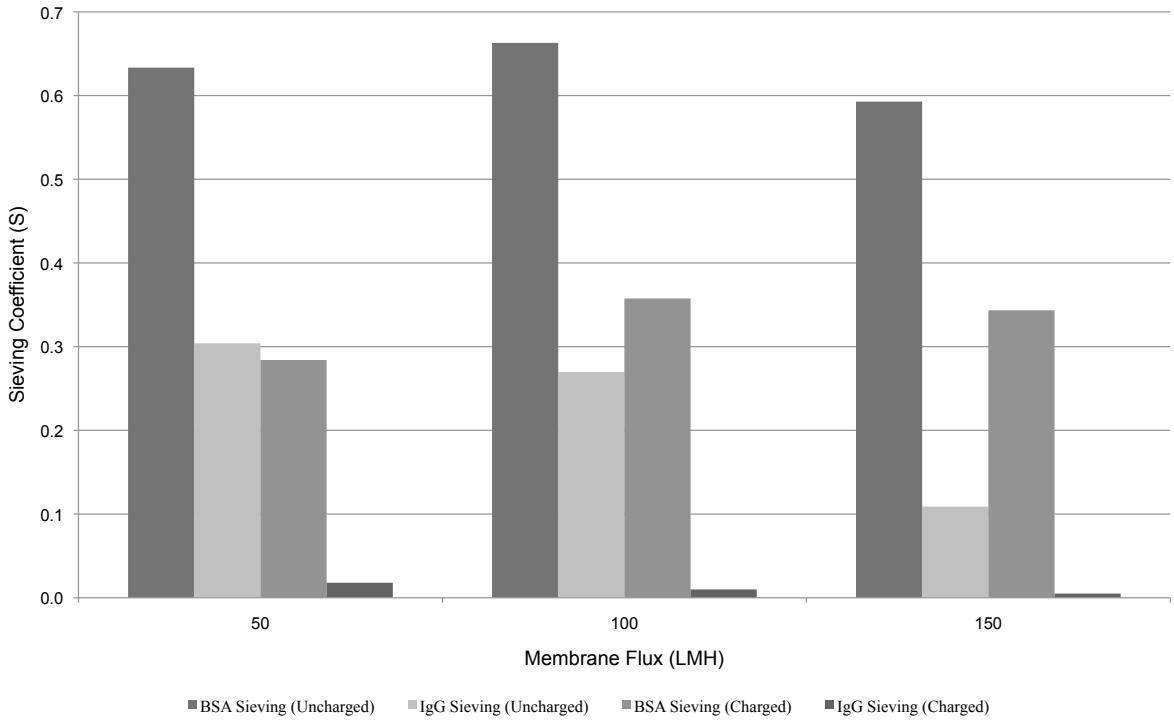


Figure 6.12: Sieving coefficients of IgG and BSA observed for the charged and uncharged variants of the 300kD Ultracel membrane at low pH and ionic strength conditions (10mM Na-Acetate, pH 4.0)

seen for BSA as a result of the decrease in membrane permeability can clearly be seen. The additional reduction in IgG sieving must therefore be due to electrostatic interactions between the IgG and the positively charged membrane.

Interestingly, the selectivity of the membrane seems to increase with increasing membrane flux. The sieving coefficient of BSA remains relatively constant across all three fluxes which were tested, whilst the sieving coefficient of IgG decreases by approximately 50% with every 50 LMH increase in flux. This increase in IgG retention with increasing flux is probably due to the same reason that the same effect was observed previously with the uncharged membrane. Increasing the membrane flux whilst maintaining the feed flow rate would have lead to increased levels of concentration polarization at the membrane surface. The build-up of positively charged molecules on an already positively charged membrane surface would have lead to high levels of electrostatic repulsion of like charged IgG molecules from the membrane resulting in the observed decrease in IgG sieving.

In order to further ensure that the improved selectivity was indeed as a result of the positive charge added to the membrane, an additional experiment was performed in which the sieving of BSA and IgG by the membrane was tested under high salt conditions. It was thought that the use of high salt concentrations would disrupt the electrostatic repulsion effects between the membrane and the positively charged IgG molecules. The pH was also increased in order to decrease the net positive charge carried by the IgG which would further reduce any electrostatic interactions which may occur.

For this experiment, the feed was IgG and BSA suspended in a buffer containing 25mM Na-Phosphate and 500mM NaCl at a pH of 7.0. This buffer was found to have a conductivity of approximately 55 mS/cm, which is more than sufficient to disrupt any electrostatic effects which may occur between the membrane and the feed components. Experiments were performed in the same manner as previously described, using the same conditions. Table 4 summarizes the results obtained.

Table 6.14: Sieving coefficient and selectivity of charged membrane in 25mM Na-Phosphate, 500mM NaCl, pH 7.0

Flux (LMH)	BSA Sieving Coefficient (S_1)	IgG Sieving Coefficient (S_2)	Membrane Selectivity (Ψ)
50	0.25	0.13	2.02
100	0.26	0.15	1.74

The results in Table 6.14 indicate that the increase in ionic strength and pH has resulted in a significant drop in membrane selectivity. This decrease in selectivity is due predominantly to an increase in IgG sieving. Comparing the results in Table 6.14 to those shown in Table 6.12, it can be seen that the BSA sieving has dropped slightly (from approximately 0.28-0.36 to 0.25-0.26). This decrease in BSA sieving is most likely due to an increase in the hydrodynamic radius of the BSA molecules as a result of the increase in charge density caused by increasing the pH past the pI of BSA. A similar result was seen with the uncharged membrane, however the decrease in BSA

sieving is relatively small compared to the almost 10-fold increase in IgG sieving seen as a result of increasing the ionic strength conditions. As a result the membrane selectivity dropped to an average value of 1.88 across the two flux values of 50 and 100 LMH. This value of membrane selectivity is highly comparable to those obtained for both sets of physicochemical conditions with the uncharged membrane.

Figure 6.13 shows the change in sieving coefficients of IgG and BSA for the charged and uncharged membranes at neutral pH conditions. These results show a drop in the sieving of both BSA and IgG with the charged membrane. This decrease is again attributable to the reduction in the effective pore size of the membrane as a result of charge addition. The important fact to note is that the percentage reduction in sieving for BSA and IgG is equivalent as opposed to the disproportionate change observed at the low pH and ionic strength conditions.

As was the case with the uncharged membrane at neutral pH conditions, it was not possible to achieve a membrane flux of 150 LMH, without experiencing an infinite increase in transmembrane pressure. As was the case previously, this is most likely due to the formation of an impermeable gel layer at the membrane surface, resulting from excessive concentration polarization. The reason for this gel layer formation is, as was the case with the uncharged membrane, due to the removal of electrostatic repulsive forces from the membrane surface, leading to more rapid fouling of the membrane. Sieving coefficients of IgG and BSA observed for the charged and uncharged variants of the 300kD Ultracel membrane at neutral pH and high ionic strength conditions (25 mM Na-Phosphate, pH 7.0).

The results of these experiments at high ionic strengths show that the performance of the membrane can be returned to that which was observed when it was in its uncharged state, by the disruption of electrostatic interactions. This provides further evidence that the improved membrane selectivity observed under the low pH conditions was due to the positive charge added to the membrane.

Figure 6.14 and 6.15 show a comparison of the changes in membrane selectivity as a result of adding a positive charge to the membrane under the two different sets of physicochemical conditions used. Figure 6.14 shows the dramatic improvement in

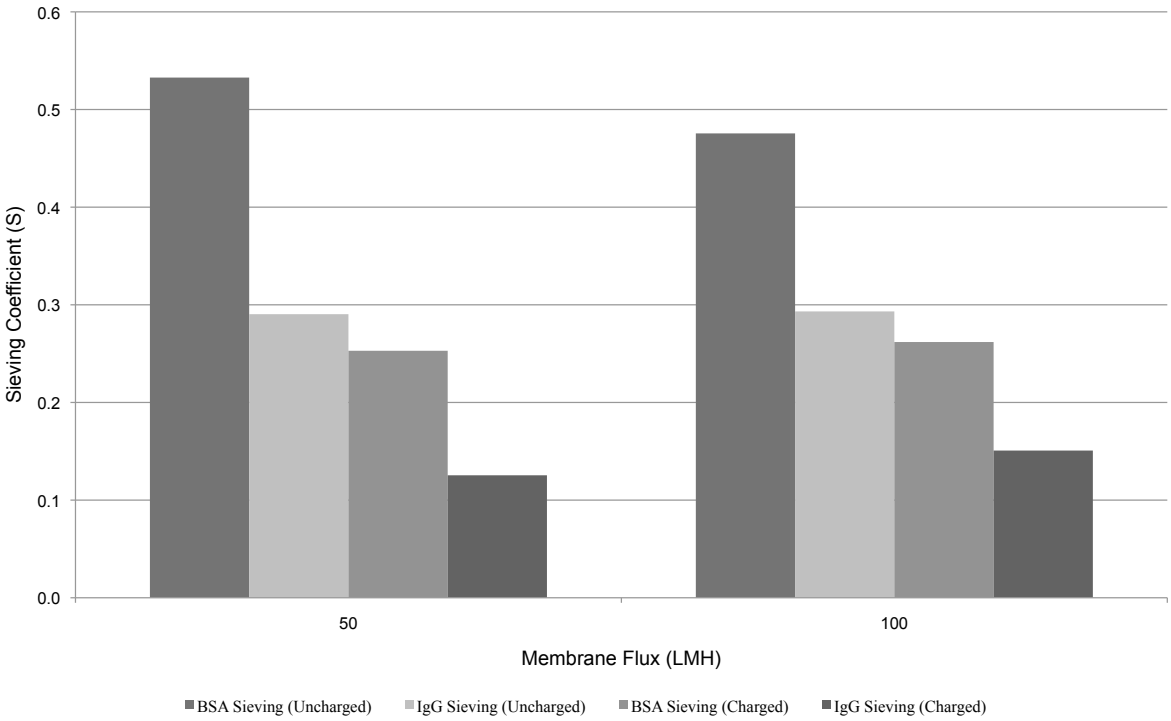


Figure 6.13: Sieving coefficients of IgG and BSA observed for the charged and uncharged variants of the 300kD Ultracel membrane at neutral pH and high ionic strength conditions (25 mM Na-Phosphate, pH 7.0)

selectivity at low pH and ionic strength conditions, whilst Figure 6.15 shows that the selectivity of the membrane can more or less be returned to that observed with the uncharged membrane by increasing the ionic strength and disrupting the electrostatic interactions between the membrane and the IgG.

6.7.6 Purification Factor and Yield of Ultrafiltration with Charged Membrane

Figure 6.16 shows the optimization diagram for the positively charged membrane under the conditions tested. This diagram shows the dramatic improvement in achievable yield and purification factors and the increase in membrane selectivity has resulted in a vastly more desirable level of trade-off between yield and purity

Whereas with the uncharged membrane, exemplified in Figure 6.16 by the lines representing membrane performance at 50 and 100LMH at pH 7.0, it was not possible to achieve purification factors of greater than 2 fold without sacrificing 50% yield,

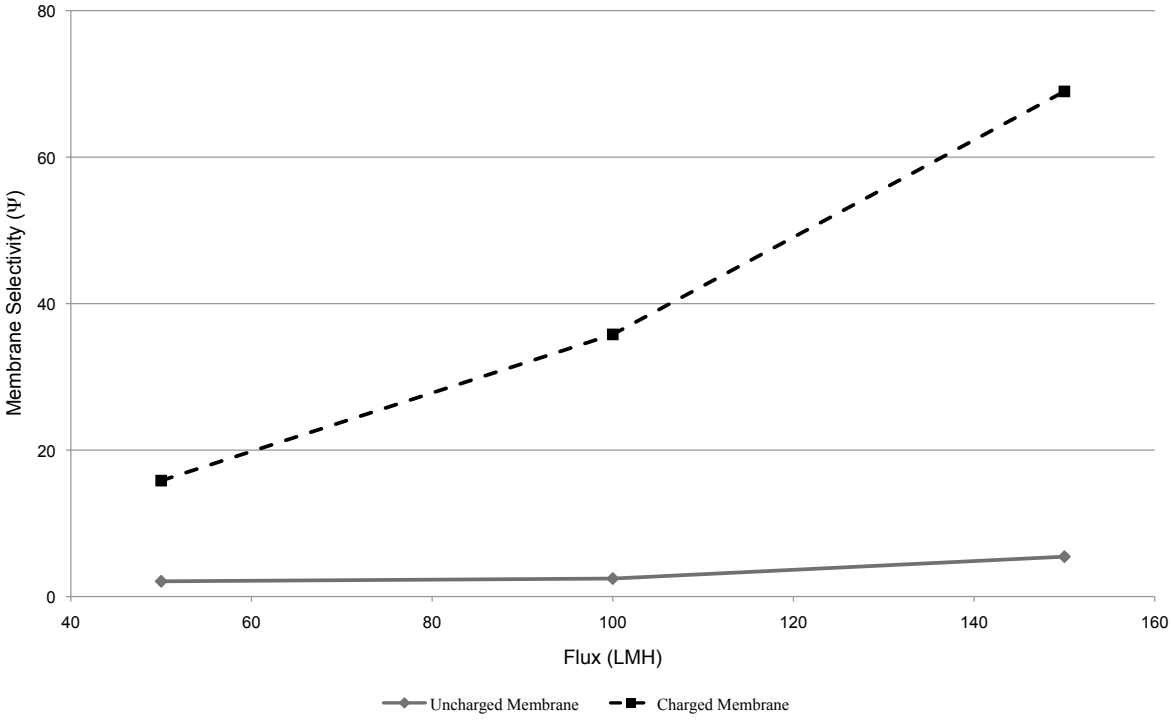


Figure 6.14: Change in membrane selectivity at low pH and ionic strength conditions (10mM Na-Acetate, pH 4.0)

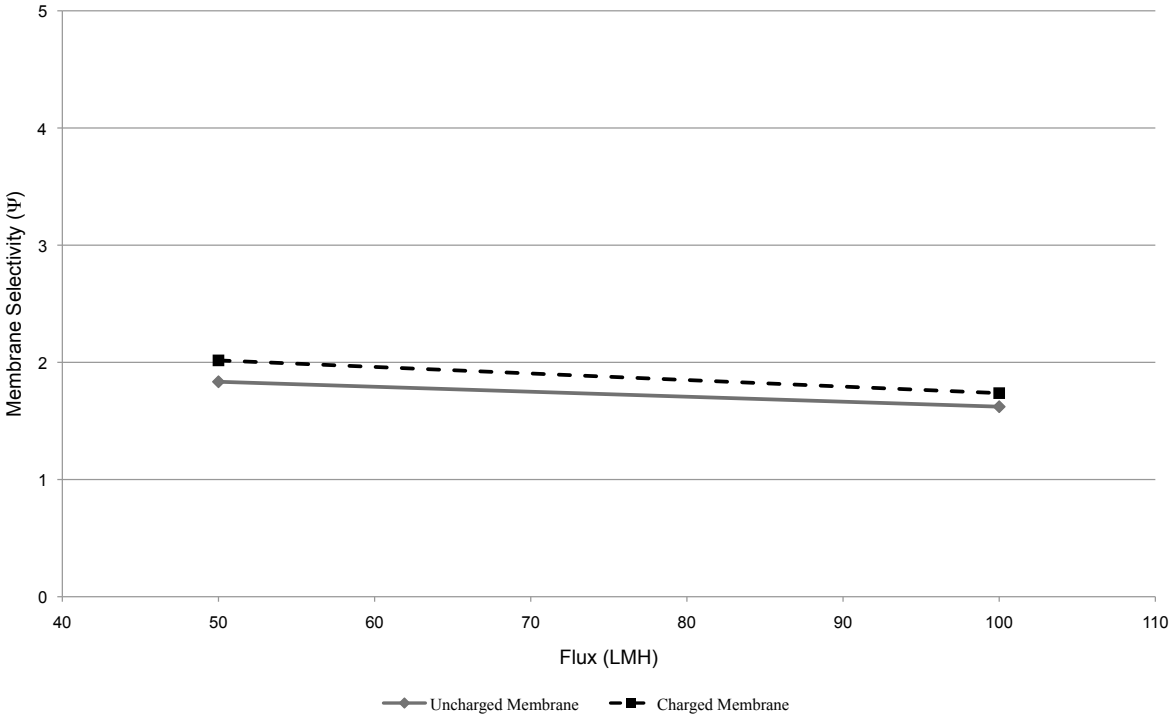


Figure 6.15: Change in membrane selectivity at neutral pH and high ionic strength conditions (150mM Na-Phosphate pH 7.0 and 25mM Na-Phosphate, 500mM NaCl pH 7.0)

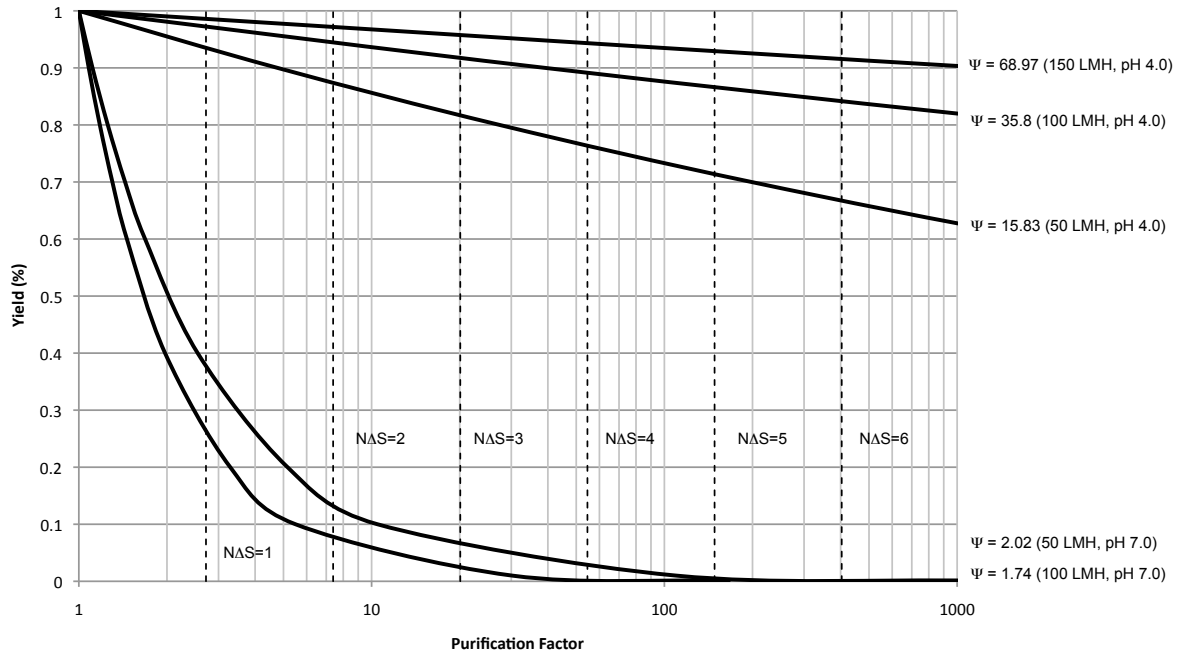


Figure 6.16: Optimisation diagram for an charged membrane used for the purification of IgG from a mixture with BSA

with the positively charged membrane operating at low ionic strength conditions it is possible to achieve purification factors of greater than 20 fold with yields still greater than 80%.

6.7.7 Normalised Water Permeability of Charged Membrane

The normalized water permeability of the charged membrane was observed to decrease dramatically after its first usage, falling from approximately 29.3 LMH/psi to 22.2 LMH/psi. An additional wash step with 0.5M NaOH did not seem to improve the NWP. However the NWP was observed to remain at this level (21.1 LMH/psi) following the second protein separation experiment. Figure 6.17 shows the change in NWP over the course of all the experiments performed on the Pellicon XL 50 Ultracel 300kD membrane.

Datapoints 1 through to 5 have already been described when discussing Figure 6.11. Datapoint 5 was the NWP measured immediately following membrane charg-

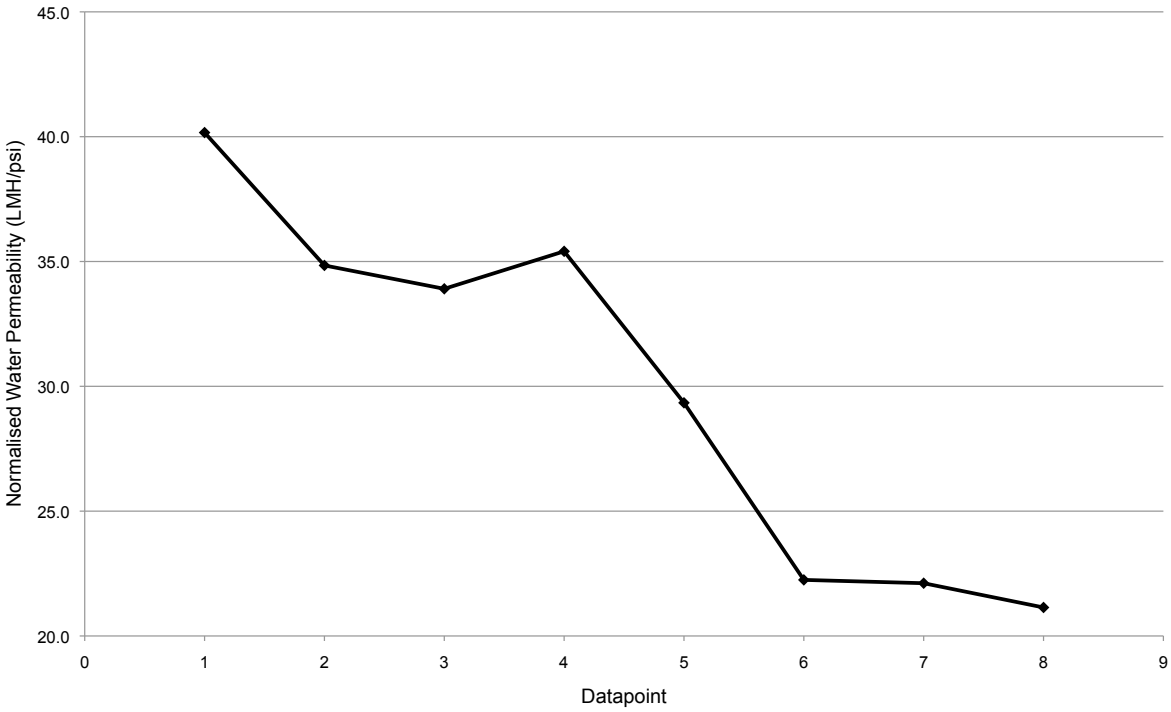


Figure 6.17: Change in Normalised Water Permeability (NWP) of the membrane over the course of experiments performed

ing. Datapoint 6 represents the NWP measured after the first protein separation experiment on the newly charged membrane. The dramatic drop in NWP observed between Datapoints 5 and 6 is most likely to be analogous to the drop in NWP observed between Datapoints 1 and 2. That is, it is caused by the initial fouling of a fresh membrane surface.

6.8 Conclusions

The aim of these preliminary experiments on ATPE and HPTFF processes was to evaluate and characterise the performance of these techniques, such that a sufficient level of understanding of their behaviour could be obtained. This knowledge could then be used to enable their effective integration into a three step mAb purification process.

The experiments performed upon the ATPE system developed by Andrews et al., showed a significant deviation from the expected partitioning behaviour of the product

antibody. These deviations could potentially be due to the characteristics of the feed material, and their relative incompatibility with the ATPE system. Alternatively, the deviations observed may point to inherent issues regarding process robustness associated with all ATPE processes. In reality, it is likely to be a combination of both of these factors. The partitioning behaviour of an ATPE system is dependent upon interactions between the characteristics of both the feed material, and those of the ATPE system itself. It is therefore not surprising that the performance of the ATPE system should deviate when the feed material is changed from that with which the system was originally developed. For example the target antibody in this case may display a greater degree of hydrophobicity than that which was originally used by Andrews et al. What is surprising however is the degree of deviation. It might be expected that the yield would decrease during the back extraction as a result of the change in feed material, but for the recovery in the bottom phase of the back extraction system to drop to zero, with the product collecting in the form of an interfacial precipitate between the two phases would indicate a significant degree of incompatibility between the ATPE system and the product antibody. All of this ultimately indicates that the concept of a generic ATPE process, which may be applied to a variety of feed types, is one which is unlikely to be realised, even if the product being purified is of the same modality. Whilst Protein A may be used to capture antibodies from a variety of load materials, without the need for significant changes in the operating parameters, a similar approach cannot be taken with ATPE system. Instead, it would seem that a blank sheet approach would almost need to be taken in process development with each new product, with the adoption of high throughput screening methodologies for development and optimisation an absolute necessity.

Regardless, the experimental work performed did result in the development of a greater degree of understanding of this ATPE system. Whilst the system could not be made to behave in the manner in which it was desired, the trade-offs observed in terms of product purification, recovery and concentration as a result of different product recovery methods can be used to inform decisions as to how best to integrate this ATPE system into the platform process. For example recovering the product in

the top phase during the forward extraction results in high process yield and product concentrations, but comes at the expense of a product pool with a potentially high impurity composition, and also properties (i.e. high viscosity) which may complicate further processing. Alternatively use of multiple back extraction steps can result in recovery of the product in the salt enriched bottom phase, but leads to excessive product dilution as well as a low overall step yield. These trade-offs may be accounted for when integrating ATPE into the platform process, which is detailed in the following chapter.

Unlike the ATPE system, the HPTFF process performed in a much more expected manner, enabling separation of a polyclonal IgG and BSA mixture. These experiments helped to illustrate the high dependence the performance of this process has upon the electrostatic repulsive forces between the charged membrane and the polyclonal IgG product. The membrane used had a MWCO of 300kD, which is approximately twice the size of the product IgG. Thus retention of the IgG and the resultant selectivity of the membrane was entirely dependent upon these electrostatic forces. Whereas the uncharged membrane was unable to provide any appreciable purification of these two components, the charged membrane could provide a 20-fold purification of the polyclonal IgG (based on total protein content) with a step yield of greater than 80%. The performance of the charged membrane could however be returned to that observed when in its uncharged form by simply increasing the ionic strength conditions in order to disrupt the electrostatic interactions responsible for the observed increase in membrane selectivity. Based on this it would therefore seem that process stream conductivity is a key parameter in the operation of a HPTFF process, and should be maintained at a relatively low level to ensure optimal membrane performance.

The Protein A based three step platform process was also run, providing a base line against which the performance of the alternative process trains may be compared. This base case is a semi-optimised process. This may possibly explain the relatively modest yield of 69% obtained from the Mabselect SuRe capture step. Aside from this, the performance of the base case process may be considered fairly typical of a standard mAb platform process. The overall process yield was approximately 67%,

whilst the final product had a HCP content of only 2 ppm. The low overall process yield is entirely attributable to the low recovery obtained over Protein A capture. A three step mAb purification process should ideally have an overall process yield of greater than 75%. However the semi-optimised nature of the base case may make comparisons with the alternative process trains slightly fairer. The performance of these alternative process trains is detailed in the following chapter.

Chapter 7

Integration of Alternative Bioseparations Techniques into a mAb Purification Platform Process - Part II

7.1 Abstract

Experimental studies were undertaken in order to evaluate the performance of ATPE and HPTFF when used as part of a three-step monoclonal antibody purification process. A well characterised three step mAb purification process, utilising Protein A, multi-modal and anion exchange chromatography was taken as a base case. Unit operations within this base case were then replaced with ATPE and/or HPTFF. The performance of these alternative containing process trains were then compared to that of the base case, in order to gain insight as to how these techniques behave when used as part of an entire process train. The results of this study would then help address the concerns over the MADM analysis presented in Chapter 5, which evaluated these alternative techniques in isolation, rather than as part of a whole purification process, thereby potentially underestimating their true industrial attractiveness.

The experimental study itself was split into two sections. In this second section,

using the insight gained from the evaluation experiments detailed in Chapter 6, ATPE and/or HPTFF were integrated into a three-step mAb purification processes. A total of five different process sequences were tested, using different configurations of ATPE, HPTFF, multi-modal and anion exchange chromatography. Difficulties were encountered in integrating these alternatives into a three-step process, thereby revealing issues of process compatibility. Whilst the performance of the process trains incorporating the use of alternative bioseparation techniques was not able to match that of the Protein A base case platform process, it was of sufficient comparability that the technical feasibility of using ATPE and HPTFF for the purification of a mAb product has been shown.

7.2 Introduction

A study was undertaken to evaluate the performance of alternative bioseparation techniques when used as part of a whole process sequence. In Chapter 5 a MADM based framework was developed, which could be used to evaluate the industrial attractiveness of a given bioseparation technique. This framework was then used to determine the relative attractiveness of a number of alternative bioseparation techniques, compared to conventional packed bed chromatography. The results of this analysis showed that none of the alternatives could match the industrial attractiveness of chromatography and as a result could not be considered viable alternatives to their more conventional packed bed counterparts. Evaluation of these techniques using the MADM framework was based on the utilisation of qualitative data gathered from relevant literature sources. As was described previously in Chapter 6, a potential shortcoming of using literature derived data, is that such information may not necessarily reflect the true potential of many of these alternative techniques, particularly for the purposes of mAb purification, where Protein A chromatography is so dominant. Another potential drawback of the MADM based methodology described in Chapter 5 is that the techniques are all evaluated in isolation, rather than as part of a whole process sequence. This may again, hide some of the true potential of these

alternative techniques.

In order to address this, ATPE and HPTFF, the two most industrially attractive alternative bioseparation techniques identified by the MADM based framework, were evaluated as part of a process sequence. These two techniques were taken and integrated into an existing mAb purification platform process, with the performance of the whole process measured against that of the standard alternative-free process. Performing such a comparison would allow the the feasibility of adopting these alternative techniques into actual purification processes to be better understood. As stated, in the MADM based evaluation, these techniques were analysed in isolation, and very little consideration was made towards how easily these alternatives could be incorporated into an actual purification process. In terms of a whole process train, the compatibility of techniques can oftentimes be a fairly subtle process characteristic, and is one which is difficult to discern without actual hands-on experience with the techniques. Thus it was hoped that by performing these whole process experiments, such aspects could be disseminated.

It was also hoped that by performing these studies, sufficient data could be obtained regarding the performance of these techniques, when used for the purification of an antibody product. Such data could then potentially be used to re-inform the process models used for the MADM based analysis, which in turn would address the concerns that the study detailed in Chapter 5 was based purely on literature data.

Four different process trains were evaluated in this study. The first process train evaluated, was the base case process, which did not contain any alternative bioseparation techniques. This base case instead reflected what could be considered a typical mAb purification platform process, with Protein A chromatography used for primary capture, followed by multi-modal anion exchange chromatography for intermediate purification and finally anion exchange chromatography for polishing. The performance of this base case process was described in Chapter 6. Also described was the evaluation and development of an ATPE and HPTFF process for the purification of a monoclonal antibody product. The results of this evaluation work revealed how sensitive the performance of these alternative techniques were to the conditions of the feed

material. This was particularly true of the ATPE process in which it was not just the performance in terms of yield and purification which was affected, but also the fundamental behaviour of the system itself. Such insight could not be obtained simply from relevant literature, and as a result provided a useful platform upon which to make further judgements regarding the ways in which these alternative techniques should be operated. Indeed, using the information gathered from this work, it was possible to develop suitable strategies for the integration of these alternative techniques into the standard platform process.

The most suitable point for integration of these techniques into a platform process, was determined based on an evaluation of their characteristics. ATPE, with its ability to handle crude feed stocks, and relatively low resolving power was thought to be most suited as a primary capture step and as a result was employed as a replacement for Protein A affinity chromatography. HPTFF meanwhile was felt to be unable to handle such crude feedstocks due to the high probability of membrane fouling and as a result was not evaluated as a primary capture step. Instead, the potential for achieving high purification factors meant that it was felt that it would be more suited for use as an intermediate purification step. Based on this, three alternative process trains were generated, as shown in Figure 6.2 in Chapter 6, and designated Process Train 2, Process Train 3 and Process Train 4 (Process Train 1 was the base case process, the characteristics and performance of which has been previously described in Chapter 6). This chapter describes the performance of these alternative process trains, and how they compared to the standard Protein A platform process.

7.3 Methods and Materials

The methods and materials used for this portion of the study were identical to those described in Chapter 6.

7.4 Results and Discussion

7.4.1 Process Train 2

In Process Train 2, the Protein A step present in the base case process is replaced by ATPE. This is then followed by an intermediate purification step using Capto adhere run in flowthrough mode and then finally a final polishing step using Capto S, also run in flowthrough mode.

Integration of ATPE into Process Train 2

Integration of ATPE into Process Train 2 was found to be problematic on a number of counts. These issues were primarily as a result of the difficulty encountered in recovering the product IgG in the salt enriched bottom phase during the back extraction step of the ATPE process, as was previously detailed. Of the many alternative approaches investigated, one which showed promised was the use of multiple back extraction steps.

Loading of Bottom Phase from Multi-step back extraction

As previously discussed, the multi-step back extraction approach, allowed product to be recovered in the bottom salt rich phase of the aqueous two phase system, through the utilisation of three separate back extraction steps. Following forward extraction, the top phase is recovered and contacted with the back extraction buffer in order to form the first two phase back extraction system. During this first back extraction, the product remains in the top polymer enriched phase. This phase is then recovered and contacted with fresh back extraction buffer in order to form a second two phase back extraction system. In this second system, a proportion of the product actually partitions into the bottom phase. The top phase however still contains a significant amount of product and so is recovered and contacted with more back extraction buffer to form a third and final two phase back extraction system. In this final system, almost all of the product partitions into the bottom phase. The bottom phases from the second and third back extraction systems may then be pooled and passed on for

futher processing. Previously development work showed that such an approach could provide reasonable product recoveries. Thus one option for integration of the ATPE process into Process Train 2 would be to simply load the pooled bottom phases from the second and third back extraction steps onto the Capto adhere column. However such an approach has a number of drawbacks.

- *Multiple Stages Compromises Process Productivity and Cost* Whilst the use of multiple back extraction steps resulted in acceptable levels of product recovery, the use of multiple back extraction stages had several implications upon the cost and productivity of the ATPE step. Firstly the use of multiple back extraction steps obviously has a deleterious effect upon the productivity of the process, as multiple rounds of phase formation, separation and recovery increases the total batch time for the step. Furthermore the requirement for high salt concentrations in the back extraction buffer increases the cost of the process overall.

- *Product Dilution* The impact on product concentration can also not be discounted. Following ATPE, Process Train 2 comprises two packed bed chromatography processes, neither of which are operated in bind and elute mode. As a result, following ATPE there are no further opportunities for product concentration. The fact that use of the multi-step back extraction approach results in an approximately 2-fold dilution of the product, over its original concentration in the cell culture supernatant means that the remaining chromatography steps are processing larger volumes of feed material than the primary capture step. This is clearly not a desirable option, particularly since in flow through mode chromatography, the bottleneck for productivity is the loading stage. Increasing the load volume can therefore have significantly deleterious effects upon the productivity of the process.

- *Clearance of PEG* As described previously, the volume of the top phase in the final back extraction step was found to be negligible. Since the product is recovered in the bottom phase of the second and third back extraction step, clearance of PEG from the system only occurs during the first back extraction, in

which some PEG will move into the bottom salt rich phase which is not collected. The remaining PEG, present in the top phase from the initial forward extraction will be predominantly distributed into the product containing bottom phase of the second and third back extraction step, with presumably only a small amount present in the top phase of the final back extraction step, due to its small volume.

- *High Conductivity* Due to its salt rich nature, the pooled, product containing bottom phases from both the second and final back extraction steps, will have a high conductivity. Even though multi-modal resins are designed to be salt tolerant to a certain degree, this most likely makes loading of this product pool directly onto the Capto adhere step following ATPE in Process Train 2 unfeasible, since high conductivities will compromise the ability of the multi-modal resin to bind impurities. Dilution of the product pool to reduce the conductivity is not a desirable option as it would involve further increasing the process volume of what is already a relatively dilute product stream.

Loading of Top Phase from Back Extraction

In light of the difficulties encountered in trying to recover the product in the salt enriched bottom phase of the back extraction aqueous two phase system, an alternative approach was proposed. Rather than utilising additional steps to try and back extract the product, it was posited that it would be more straightforward to simply recover the product enriched top phase, following the first back extraction, and take this for further processing and purification. Taking such an approach would not eliminate all of the previously described problems, associated with loading pooled bottom phases from a second and third back extraction step, but would help to address the issues of process productivity and also product dilution. Elimination of the second and third back extraction stages not only decreases the yield losses encountered as a result of incomplete phase recovery, but also reduces the associated batch time for the ATPE process, thereby increasing the overall process productivity. Furthermore it also decreases the level of product dilution encountered which helps to increase

the productivity of the subsequent packed bed chromatography steps. The issues of high conductivity and high PEG concentration however remain, since NaCl partitions equally into both top and bottom phases of the two phase system. The high PEG concentration is of a particular concern, since the top phase of the first back extraction step is very viscous. Loading such material onto a chromatography column would potentially result in unacceptable system pressures, as well as the possibility of viscous fingering within the packed bed. It was decided that of the two possible approaches, loading the top phase from the first back extraction onto the Capto adhere step was the more suitable options, as it presented fewer drawbacks than using a multi-step back extraction. The high conductivity and also PEG composition of this top phase meant that it was necessary to employ a diafiltration step, between ATPE and Capto adhere. However such a step would also have been necessary if the multi-step back extraction approach had been utilised. The top phase from the back extraction system was chosen over the top phase from the forward extraction, since based on the results obtained, the first back extraction does provide some degree of purification, with impurities partitioning into the bottom phase.

Process Train 2 - ATPE

The aqueous two phase forward extraction was performed by adding PEG 1500, K_2HPO_4, NaH_2PO_4 and NaCl powders directly to the cell culture supernatant feed, so as to achieve a final system composition of 15% (w/w) PEG 1500, 14% (w/w) Phosphate and 12% (w/w) NaCl, with a system pH of 6.0. A 27% (w/w) sodium citrate back extraction buffer was added to the recovered top phase from the forward extraction in a top phase to citrate buffer volume ratio of 1 to 0.81, resulting in a back extraction system with a citrate concentration of approximately 15% (w/w). The back extraction systems were mixed, incubated and then phase separated using centrifugation. The product containing top phase from the back extraction was then diluted 1 in 4 with 60mM sodium citrate buffer, pH 3.4 to achieve low pH viral inactivation. Dilution of the top phase in this manner caused a precipitate to form in the product pool which was removed using centrifugation. The diluted top phase was

then further diluted by 1 in 2 with 25mM Phosphate, pH 6.5, before being titrated to pH 5 using 0.1M NaOH in order to neutralise the low pH treated product pool. The product recovery following the back extraction stage of the ATPE process was approximately 86%, and provided a 6 fold reduction in HCP to IgG content.

Process Train 2 - Diafiltration

The diluted and neutralised top phase was then concentrated by a factor of approximately 8-9 fold, in order to return the product pool volume to that of the original top phase from the back extraction and prior to dilution and neutralisation. Following concentration the product pool was then diafiltered into 25mM Phosphate, pH 6.5, using a total of approximately 7 diavolumes. Both the concentration and diafiltration were performed using a Kvick Start membrane (GE Healthcare, Uppsala) with a molecular weight cut off (MWCO) of 10kD. This pore size was deemed large enough so as to allow clearance of PEG 1500 from the retentate, whilst also small enough to ensure that the diafiltration step did not result in a high level of product transmission into the permeate. The membrane was operated at a crossflow rate of 300L/m^2/h and a transmembrane pressure (TMP) of 1.5 bar. A total membrane load of approximately 46g IgG per m^2 was used.

The diafiltration process resulted in an exceptionally low process yield of approximately 30%, even with membrane buffer flushes, used to maximise product recovery. Protein A analysis of permeate samples revealed only a negligible amount of protein transmission through the membrane, as would be expected given the relatively small MWCO used. It should be noted at this point that a large amount of precipitation was observed in the retentate during the diafiltration process. Slight increases in the turbidity of the retentate would be expected during concentration of an IgG product, however such changes are often only observed once the protein concentration rises above 25g/L. In this case, the IgG concentration should not have reached levels any greater than 5g/L (the IgG concentration of the top phase following back extraction was 4.8g/L). Whilst recovery and analysis of this precipitate was not performed, given the negligible amount of IgG present in the permeate, it can be assumed that

the large amount of precipitation observed during the diafiltration process was indeed IgG product dropping out of solution, explaining the highly modest yield obtained from this particular process step. Furthermore, it is likely that the precipitate was also partially composed of HCP, since the concentration of this impurity relative to the concentration of IgG remained almost constant, at approximately 2,700 ppm, across the diafiltration process, despite the decrease in antibody yield.

The precise cause of this precipitation can only be speculated upon, as further work to characterise the phenomenon was not performed. However it might be reasonable to assume that the presence of PEG and kosmotropic salts such as phosphate and citrate may have lead to critical reductions in protein solubility. For example whilst the membrane had a MWCO of 10kD, thereby theoretically allowing unhindered transmission of PEG 1500 into the permeate, it is more likely that the non-monodisperse nature of the membrane pores would have lead to some degree of PEG concentration polarisation at the membrane surface. This would have lead to the formation of localised areas of low protein solubility. Product IgG entering such areas could conceivable come out of solution, resulting in the observed precipitate formation.

Process Train 2 - Capto adhere

Following diafiltration, the product pool was loaded onto a Capto adhere column operated in flowthrough mode. The load concentration in this case was 37.5g/L resin, which is significantly lower than the IgG load for the Capto adhere step in the base case process. The loading capacity was lowered to account for the 6-fold higher HCP content of the product pool following ATPE and diafiltration compared to that of the Protein A eluate in the base case. It was expected that matching the load concentration for Capto adhere in the baseline process would most likely have resulted in some level of impurity breakthrough in this case, and as a result the load concentration was reduced. The flowthrough from the Capto adhere step was fractionated and analysed using Protein A chromatography in order to determine both the IgG content and also the "impurity" profile during the course of the loading process, based on the peak areas of both components on analytical Protein A chromatograms. "Impurities" in

this case refers to any process stream components which do not bind to Protein A and also exhibit UV absorbance at a wavelength of 280nm.

Figure 7.1 shows the breakthrough of IgG on the Capto adhere column, based upon the IgG concentrations of both the Capto adhere load and that of the flowthrough fractions. The relatively shallow shape of the breakthrough curve reflects the weak partitioning mode in which the Capto adhere step is being run. Furthermore, complete product breakthrough is not reached until approximately halfway through the loading process. As described previously, even though it is being run in flowthrough mode, the conditions under which the Capto adhere step is being operated means that there will always be a certain level of IgG binding.

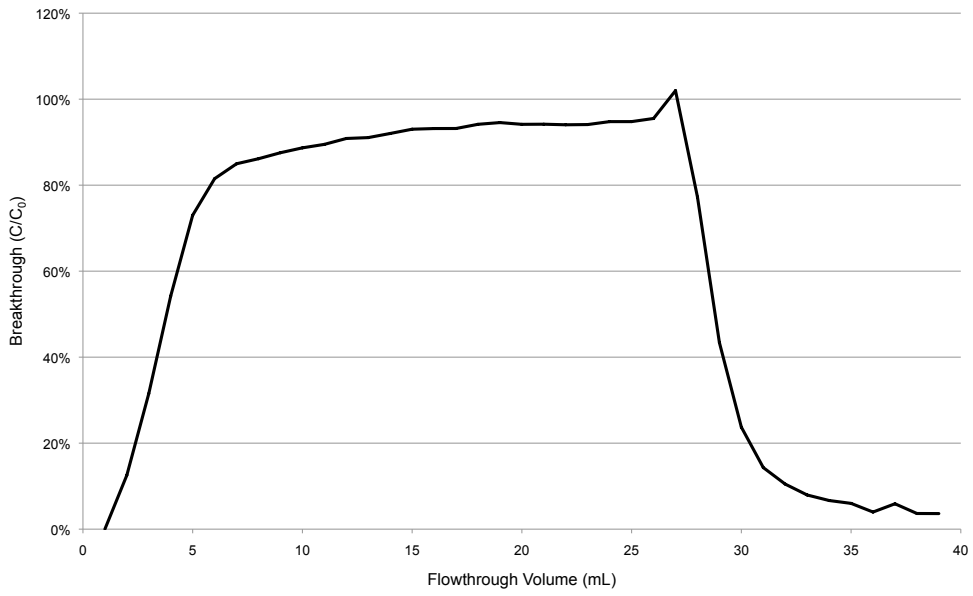


Figure 7.1: Chart showing the breakthrough of IgG in the flowthrough from the Capto adhere step of Process Train 2 during the loading stage of the process

Figure 7.2 shows the breakthrough of "impurities" on the Capto adhere column, based upon the area of the "impurities" peak on the Protein A chromatogram of the Capto ahere load material, and that of the flowthrough fractions. The precise composition of this "impurities" component of the Capto adhere load is unlikely to be the same as that in each of the flowthrough fractions. As a result, the breakthrough

values shown in Figure 7.2 do not represent the absolute levels of impurity breakthrough, instead the data can be used to provide an impression of the relative level of breakthrough of non-IgG components over the course of the loading stage of the process. From Figure 7.2 it is apparent that Capto adhere is not capable of binding all proteinaceous impurities in the process stream. Breakthrough occurs almost immediately, with the level of breakthrough steadily increasing until the end of the loading process (after approximately 24.5mL). Whilst Capto adhere does not bind all impurities present in the process stream, it does seem to have removed some components as evidenced by the fact that all the breakthrough values shown in Figure 7.2 are lower than 40%.

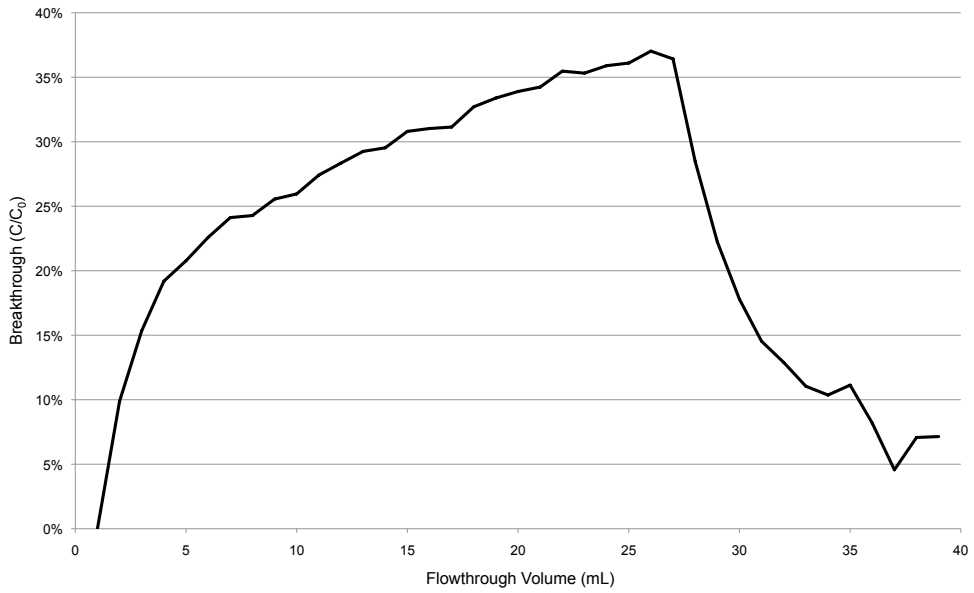


Figure 7.2: Chart showing the breakthrough of "impurities" in the flowthrough from the Capto adhere step of Process Train 2 during the loading stage of the process

Figure 7.3 shows the product recovery and corresponding product purity as a function of the flowthrough volume collected. In this case the product purity was calculated based on the relative areas of the impurity and IgG peaks on the analytical protein A chromatograms. Based on the information presented in Figure 7.3, collecting the first 20mL of flowthrough would have resulted in a product pool with

a purity of 90%. This means that the cumulative areas of the impurity peaks from the first 20mL of flowthrough fractions was 10% of the area of the IgG peaks across the same fractions. Whilst this purity figure has no "real-world" meaning, presenting this data in the manner shown in Figure 7.3 gives a good impression of the trade-off between product purity and yield. Increasing the volume of the flowthrough collected increases the product yield, however since impurity breakthrough continually increases throughout the loading step, the purity of the flowthrough also decreases.

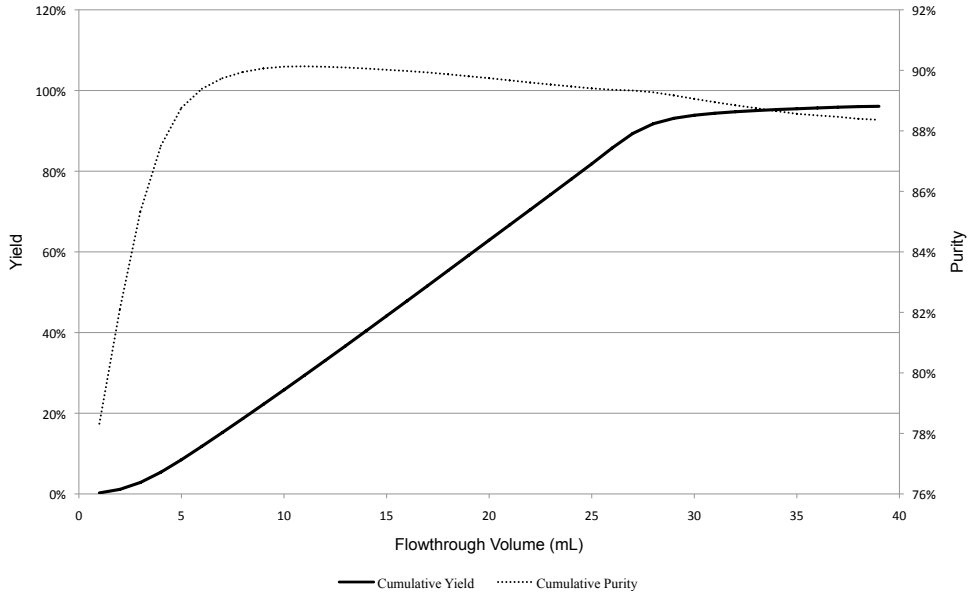


Figure 7.3: Chart showing the cumulative yield and purity achievable through pooling of different volumes of flowthrough from the Capto adhere step in Process Train 2

From Figure 7.3, it would seem that the process yield plateaus at a value of between 88-89% at a flowthrough volume of approximately 27.5mL. The product purity, after an initial sharp rise steadily decreases over the course of the load, however at the point at which the yield plateaus, the rate of purity decrease also seems to increase. This point then would seem to be an ideal location at which to stop collection of the flowthrough from the Capto adhere process, in order to maximise both process yield and also product purity. Collection of the first 27.5mL of flowthrough from the Capto adhere column, resulted in a product with an IgG concentration of 1.54g/L

and a HCP content of 1,023 ppm. The product pool from the Capto adhere step was buffer exchanged using a desalting column, as in the base case process, in order to get the product in the Capto Q equilibration buffer. In the base case process, it was observed that a significant level of HCP clearance was achieved using the desalting column. A similiar level of purification was not obtained in Process Train 2. The HCP concentration of the Capto adhere flowthrough was approximately 1450 ng/mg IgG (ppm). The flowthrough from the desalting step, and the load for the Capto Q step had a HCP concentration of approximately 800 ppm. The desalting step therefore did provide an approximate 1.8 fold reduction in HCP concentration. However in the base case, the HCP reduction afforded by the desalting step was greater than 19 fold.

Process Train 2 - Capto Q

After buffer exchange using the desalting column, the product pool was loaded onto the Capto Q column at a concentration of 19.5 g/L resin. As was the case with the Capto adhere step, this loading concentration is significantly lower than the 142 g/L resin loaded onto Capto Q in the base case process. However, given the relatively higher HCP content of the load material in this case, it was hoped that lowering the load concentration would result in an increased level of purification. Again, the flowthrough from the Capto Q step was fractionated and analysed using Protein A chromatography.

Figure 7.4 shows the breakthrough of IgG over the course of the loading step on Capto Q. From this it can be seen that the level of IgG binding to the Capto Q column is significantly less than was observed with the Capto adhere step. This is evidenced by the sharper product breakthrough, with the curve in Figure 7.4 reaching a plateau after approximately 4 column volumes, compared to the 15 column volumes seen with Capto adhere.

Figure 7.5 shows the breakthrough of "impurities" over the course of the loading step on Capto Q. As was the case with the data presented in Figure 7.2, the breakthrough percentages shown here are based on peak areas from the analytical Protein A chromatograms. From this it can be seen that Capto Q does not successfully bind

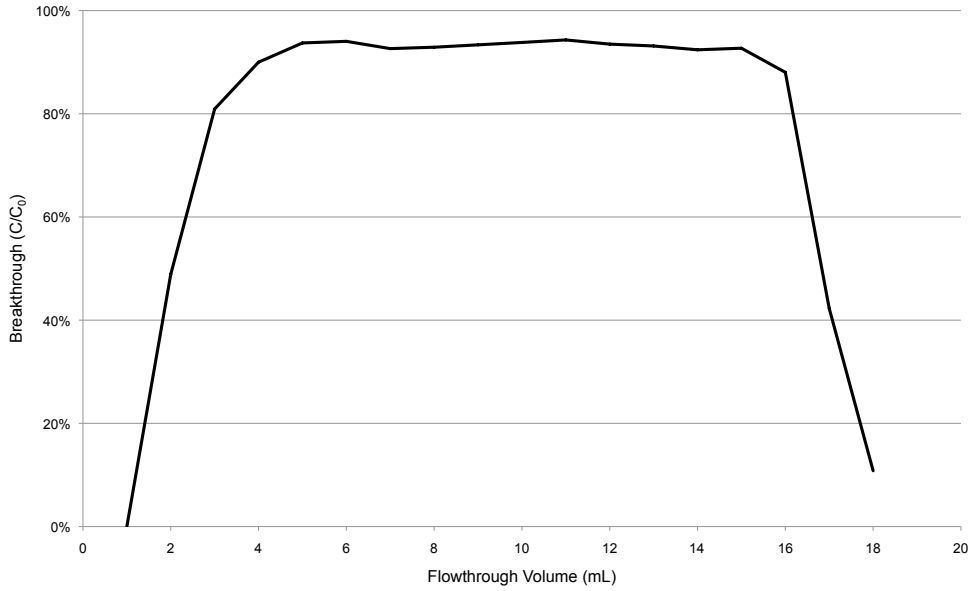


Figure 7.4: Chart showing the breakthrough of IgG in the flowthrough from the Capto Q step of Process Train 2 during the loading stage of the process

all non-IgG proteinaceous process stream components, with "impurity" breakthrough occurring at approximately the same time and rate as that of the antibody product. Unlike with Capto adhere however, the impurity breakthrough reaches a very definite plateau, which would indicate that the components breaking through have no affinity for the positively charged Capto Q ligands. This in turn would imply that it might be possible to increase the loading on the Capto Q step, without having a detrimental effect upon the purity of the product containing flowthrough.

Figure 7.6 shows the product recovery and corresponding product purity as a function of the flowthrough volume collected from the Capto Q step. Given the fact that impurity breakthrough occurs almost immediately, it is not surprising that product purity is highest at the start of the flowthrough. Collecting increasing volumes of flowthrough then only has the effect of reducing the product purity whilst increasing the product recovery.

Due to the early breakthrough of impurities on the Capto Q step, it was decided that the flowthrough collection criteria should be based purely on the achievable

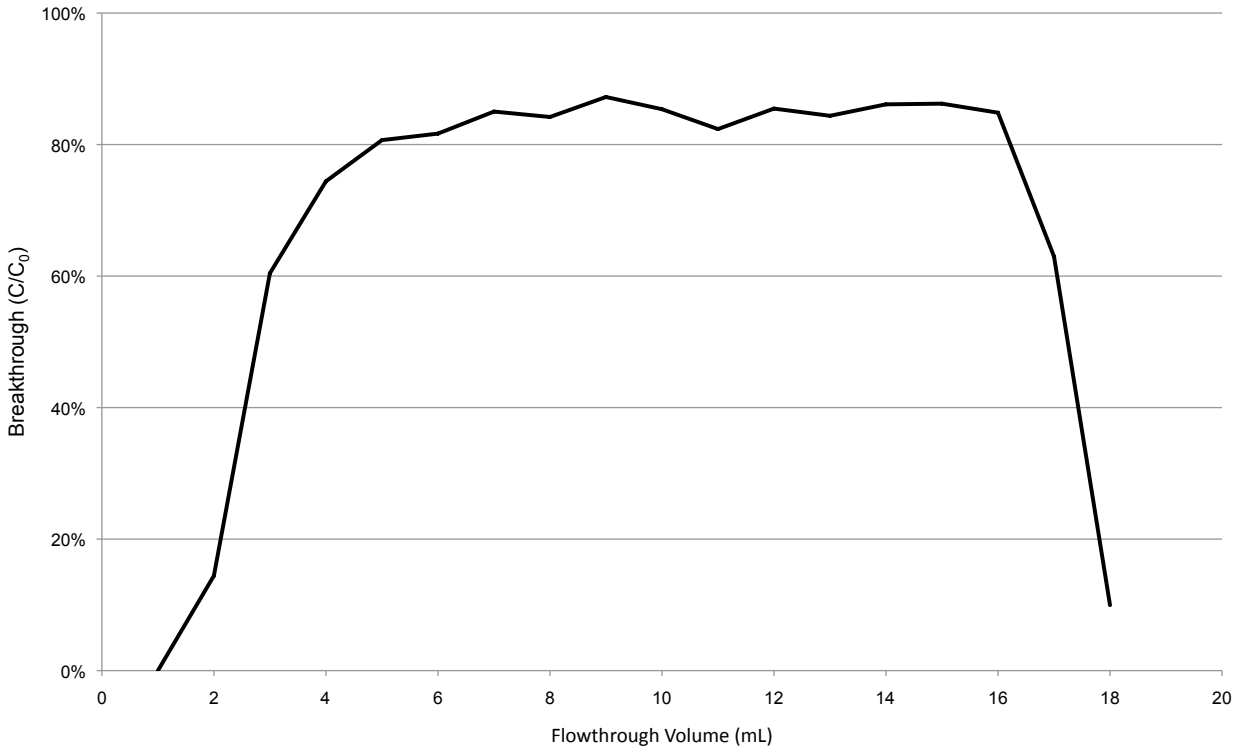


Figure 7.5: Chart showing the breakthrough of "impurities" in the flowthrough from the Capto Q step of Process Train 2 during the loading stage of the process

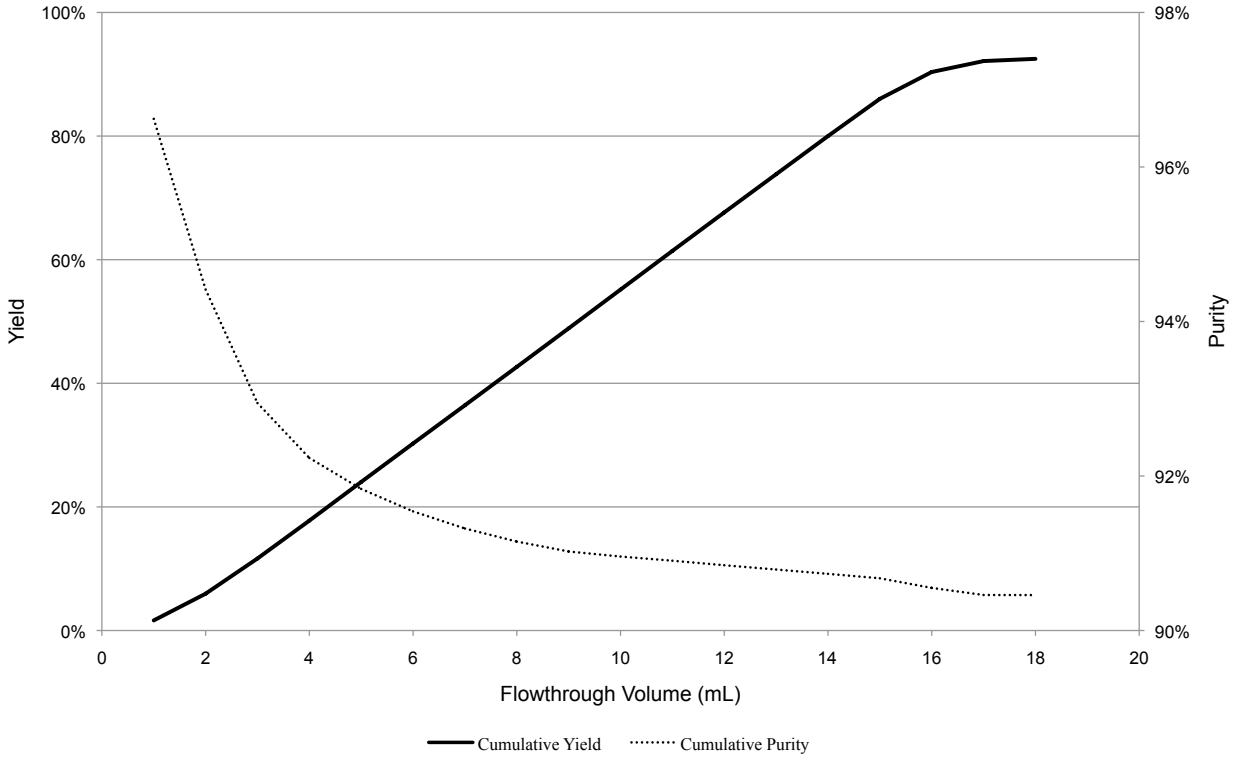


Figure 7.6: Chart showing the cumulative yield and purity achievable through pooling of different volumes of flowthrough from the Capto Q step in Process Train 2

product yield. As a result the first 16mL of the Capto Q flowthrough was collected to form the final product pool. The product pool was determined to have an IgG concentration of approximately 1.2g/L and a HCP composition of 109 ppm. Table 7.1 summarises the yield and purification performance of Process Train 2.

Table 7.1: Summary of results from Process Train 2

	Initial		Final			
Step	mAb Conc. (g/L)	HCP (ppm)	mAb Conc. (g/L)	HCP (ppm)	Yield	PF
ATPE	4.60	16,848	4.80	2,708	86%	6.22
Diafiltration	0.60	2,755	2.08	1,995	41%	8.44
Capto adhere	2.08	1,995	1.54	1,023	86%	1.95
Desalting Step	1.54	1,023	1.30	800	100%	1.28
CaptoQ	1.30	800	1.19	109	101%	7.32

Summary

Process Train 2 achieved an overall process yield of 31% which is approximately half the recovery achieved by the base case process. This low yield is almost entirely attributable to the product loss observed during the diafiltration process, following ATPE. Indeed, if the recovery from the diafiltration process could be increased to 100%, then the overall yield for Process Train 2 rises to approximately 75%, which is actually a 10% improvement over the product yield achieved using the base case process. This is mainly as a consequence of the higher recovery obtained using ATPE compared with Mabselect Protein A. However the need for diafiltration in order to integrate the ATPE process into the platform base case process, lead to a significant level of product loss.

Furthermore, the removal of Mabselect SuRe protein A from the platform process has had a significant impact upon the purifying power of the process train. The final product pool from the base case process had a HCP composition of only 2 ppm. Meanwhile the final product from Process Train 2 was found to contain HCP at a concentration of just over 100 ppm. This is a fairly high HCP content, and generally a concentration of less that 10ppm would be aimed for.[17]

The results would indicate therefore that Process Train 2 is unable to match the

performance of the base case platform process. However that is not to say that it is without some potential. The low product recovery obtained using Process Train 2 is entirely attributable to the poor performance of the diafiltration step used to integrate ATPE into the process. If it were possible to either improve or remove this step altogether from the process train, then the performance of Process Train 2 in terms of product recovery could become comparable to that of the base case process.

Similiarly whilst the HCP content of the final product from Process Train 2 is relatively high when compared to that of the base case process, it must be considered that the Capto adhere step and Capto Q steps were optimised for use in a process in which Protein A affinity chromatography was used for product capture rather than ATPE. Therefore it could be sensibly assumed that the final product purity, in terms of HCP concentration, could be improved with further process development of the multi-modal and anion exchange chromatography steps. It was concluded therefore that whilst the use of ATPE in place of Protein A in the platform process, did not provide satisfactory results, the relative performance is not sufficiently poor so as to completely rule out the feasibility of an ATPE based mAb purification train.

7.4.2 Process Train 2A

In light of the relative performance of Process Train 2, it was decided that an alternative process sequence should be evaluated, in order to further characterise the processing potential of ATPE. The diafiltration step following ATPE in Process Train 2, used to buffer exchange the product pool, in order to make it suitable for loading onto the Capto adhere step was clearly an area in which problems arose. The low flux, coupled with the observed precipitation and consequently low step recovery, meant that the overall productivity of Process Train 2 was fairly low. Furthermore, the Capto adhere and Capto Q steps combined were unable to clear HCP to a level comparable to the base case process.

From the performance of Process Train 2, it was felt that it might be possible to increase the the purity of the final product as well as the overall process yield, by replacing the Capto adhere step with a cation exchange process, run in bind and elute

mode. As previously detailed, a major causative factor for the low recovery obtained from Process Train 2 was the diafiltration step used to buffer exchange and essentially reduce the conductivity of the product containing top phase from the ATPE process, so that it could be loaded onto the Capto adhere step. The diafiltration step also served to clear the PEG used in the ATPE system from the product stream. Dilution of the top phase from the ATPE process would have potentially allowed for direct loading of the product pool onto the Capto adhere column, however the level of dilution required in order to bring the conductivity to an acceptable level would have resulted in an unfeasibly large process volume, which could not have been reduced by the Capto adhere step, operated in flowthrough mode. Indeed it was determined that at least a 1 in 32 dilution with MilliQ water would have been required to reduce the conductivity of the top phase to approximately 5 mS/cm, which would have been a compatible level for the Capto adhere step.

As a result, it was felt that an suitable alternative strategy would be to replace Capto adhere with a cation exchange chromatography step run in bind and elute mode, enabling the elimination of the diafiltration step used to integrate ATPE with the rest of the process train. The cation exchange step, operated in bind and elute mode, would be able to accomplish the concentration, buffer exchange and PEG clearance functions of the diafiltration process, thereby making its presence in the purification train, no longer necessary. Removal of the diafiltration step from the process should serve to eliminate the product precipitation issues encountered with Process Train 2 and therefore improve the overall recovery of the process. The use of a cation exchange step in place of Capto adhere could also potentially address the HCP issue encountered with Process Train 2. Operation in bind and elute mode, opens up the possibility of utilising column washing strategies in order to clear HCP from the process stream and therefore improve product purity over that obtained using Capto adhere. In this new process sequence, denoted Process Train 2A, the cation exchange resin used was Capto S (GE Healthcare, Uppsala, Sweden).

Process Train 2A - ATPE

The ATPE process was performed in the same manner as in Process Train 2, with the the top phase from the back extraction step diluted 1 in 4 with 60mM Na-Citrate, pH 3.4 buffer in order to affect viral inactivation. Following a 45 min incubation at room temperature, this viral inactivated pool was diluted 1 in 8 with MilliQ water in order to reduce the salt concentration and to thereby reduce its buffering capacity. The pool was then neutralised with 0.1M NaOH to a storage pH of 5.3. The conductivity of the product pool at this point was measured to be 5.49mS/cm, which was deemed sufficiently low in order to allow for binding of the product IgG onto the Capto S column. The yield following the ATPE and product dilution steps was measured to be approximately 90% and therefore comparable to that which was achieved using Process Train 2 following product capture (and prior to diafiltration). Similiarly the HCP composition of the product pool was measured to be approximately 2800 ppm, which whilst over five times greater than the HCP composition following Protein A in the base case, was comparable to the HCP composition following ATPE in Process Train 2. The product concentration in the top phase prior to dilution was measured to be 5.7g/L, again comparable to that which was observed following ATPE in Process Train 2.

Process Train 2A - Capto S

The Capto S step, following ATPE, was loaded to a capacity of approximately 45 g/L resin. The column was washed with 20mM Na-Citrate, 150mM NaCl at pH 3.5 and then with 40mM sodium phosphate at pH 6.5. A single column volume of equilibration buffer was passed through the column between the two wash buffers. The re-equilibration of the column between wash steps was necessitated by the fact that the pH conditions within the column were found to transition more rapidly than the conductivity. As a result, directly switching from the first wash buffer to the second, resulted in the development of high salt (150mM NaCl from the first wash buffer) and high pH (pH 6.5 from the second wash buffer) conditions, leading to premature

elution of product from the column. These effects were overcome by the one column volume re-equilibration between wash steps. Elution was performed using 100mM sodium phosphate, pH 7.0.

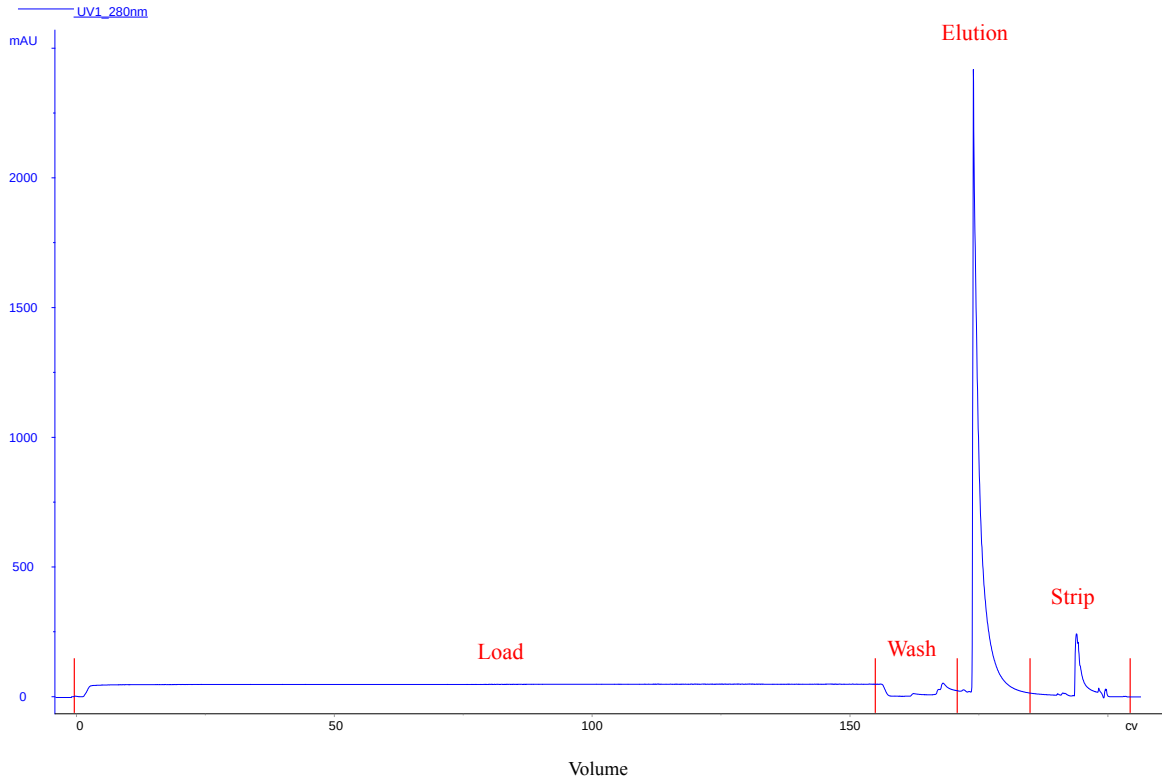


Figure 7.7: Chromatogram from the Capto S step of Process Train 2A

The eluate was collected at a UV absorbance of greater than 50mAU. Analysis of the resultant Capto S elution pool revealed an IgG concentration of approximately 1 g/L. This product recovery represented a step yield of only 15%, which less than half the recovery achieved using diafiltration in Process Train 2.

From figure 7.7, it can be seen that there is some protein present in the wash fractions of the Capto S step. However these wash peaks are not sufficiently large as to account for the 85% of lost product during this step. The chromatogram however also shows a small level of breakthrough during the loading. This was initially assumed to be HCP breaking through the column, however analysis of the flowthrough fractions showed that IgG appeared in the column output almost immediately upon commencement of loading. Indeed the entire flowthrough fraction was found to con-

tain approximately 27 mg of IgG product. Considering that the column was only loaded with 43 mg of IgG, the product lost in the flowthrough represented approximately 62% of the total IgG present in the feed.

It would therefore appear that the conditions of the feed did not sufficiently facilitate binding of the product to the cation exchange resin. The conductivity and pH of the column load were however at values which should have allowed for IgG binding. The conductivity was only 5mS/cm, which could be considered within the typical operating range for this resin, particularly when in combination with a loading pH which was well below the product pI, ensuring that the MAb was positively charged.

The low level of product binding may have been caused by the presence of some other component within the process feed. One possible explanation is that the IgG molecules were competing for binding sites with some other positively charged component in the feed stream. This could be feasible, particularly considering the relatively low resolving power of the ATPE step preceding the cation exchange chromatography process. Some positively charged HCPs could therefore have had higher electrostatic affinities for the negatively charged chromatography ligands, resulting in the low observed IgG binding capacity. Such a scenario is unlikely however, given the fact that IgG was observed to appear in the flowthrough almost immediately following the commencement of loading. Cation exchange resins typically have total protein binding capacities of at least greater than 50g/L resin. Given the low concentration of the feed material, it is unlikely that the Capto S column would have been loaded to capacity so quickly. If competive binding of HCPs over IgG were indeed the case, then the breakthrough of product would have occurred until much later on in the loading process rather than at the start, when there would have been plenty of available capacity.

Another potential reason for the low binding of IgG could be the residence time. Too short a residence time could have compromised the capacity of the Capto S column. The loading of the Capto S step was performed at a flow rate which resulted in a residence time of 2.4 minutes. Data from GE Healthcare on Capto S, suggests that the dynamic binding capacity of Capto S plateaus at a maximum at residence times beyond 2.5 minutes. Therefore the 2.4 minute residence time should not have

caused such a dramatic drop in the dynamic binding capacity.

Another possible explanation is that the product IgG has hydrophobic tendencies, with a lack of charged groups present on the protein surface. This would have resulted in a low level of product binding which in turn could explain the low product capacity which was observed.

A fourth and final possibility is that the presence of PEG 1500 in the feed stream somehow interfered with the electrostatic interactions between the product IgG and the negatively charged cation exchange ligands. It may be possible that hydrophobic interactions between the IgG and the PEG 1500 polymer molecules, could have resulted in the shielding of charged groups on the protein surface, which ties in with the theory of the antibody product having hydrophobic tendencies. This would have reduced the electrostatic affinity of the IgG molecules for the cation exchange resin, resulting in the poor binding which was observed. If this is indeed what has occurred, then it seems to be the case, as was found with process train 2, that integration of ATPE into the platform process has compromised the overall recovery of the purification train. Furthermore, the HCP composition following Capto S was found to be approximately 2000 ppm, indicating that the Capto S step has not provided any significant level of purifying power.

Process Train 2A - Capto Q

Following the Capto S step, the product pool was buffer exchanged in to 25mM sodium phosphate pH 7.5 using a HiPrep desalting column and then loaded onto a Capto Q anion exchange column, operated in flowthrough mode. It should be noted that following Capto S, the product pool was diluted 1 in 2 with the Capto S elution buffer, in order to increase the process volume. This was done in order to ease the material handling. The flowthrough from the Capto Q step was fractionated and analysed as before in order to determine both the yield and purity which could be achieved by pooling the sequential fractions. Figure 7.8 shows product recovery and corresponding purity as a function of the flowthrough volume collected from the Capto Q step. It can be seen that impurity breakthrough occurs almost immediately. The

fact that the cumulative impurity curve rises sharply and flattens out would imply that these impurity components are breaking through since they are either neutral or positively charged and hence have no affinity for the anion exchange resin, rather than because there is not sufficient capacity to bind them.

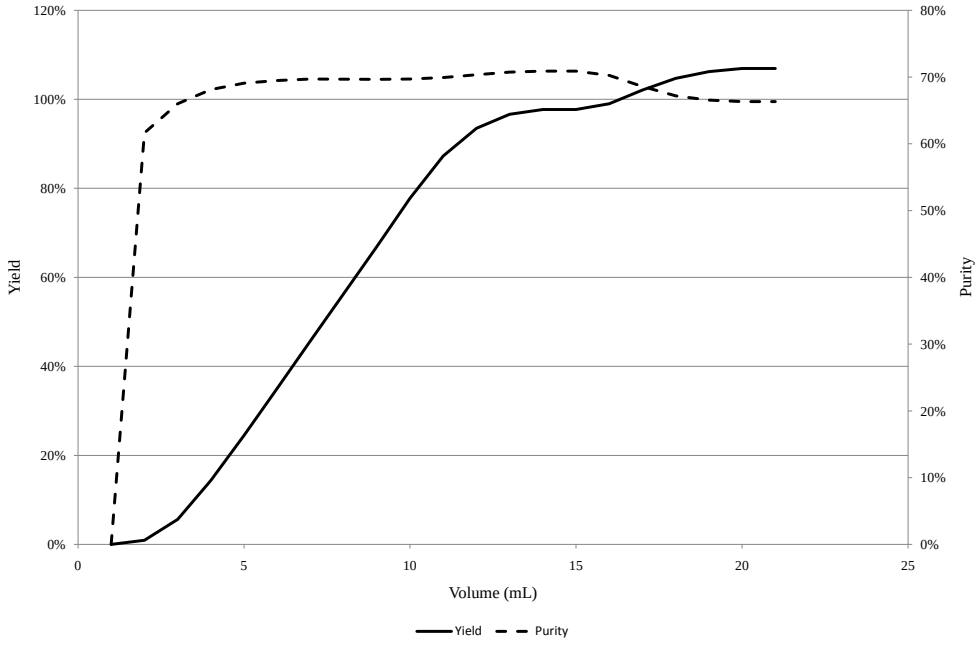


Figure 7.8: Chart showing the cumulative yield and purity achievable through pooling of different volumes of flowthrough from the Capto Q step in Process Train 2A

As was the case with the Capto Q step in Process Train 2, due to the early breakthrough of impurities the flowthrough collection criteria was based entirely upon the achievable process yield and 15mL of the flowthrough were collected to form the final product pool. This was determined to have an IgG concentration of 0.27 g/L and a HCP composition of 96 ppm. Table 7.2 summarises the yield and purification performance of Process Train 2A.

Summary

Interestingly, the Capto Q step was capable of reducing the HCP composition of the product pool by approximately 23-fold in Process Train 2A, whilst in Process Train 2, even though the load material was "cleaner", the same step was only capable of

Table 7.2: Summary of results from Process Train 2A

Step	Initial		Final		Yield	PF
	mAb Conc. (g/L)	HCP (ppm)	mAb Conc. (g/L)	HCP (ppm)		
ATPE	4.60	16,848	5.70	2,789	91%	6.04
Capto S	0.29	2,759	0.97	2,165	15%	1.27
CaptoQ	0.47	2,234	0.27	96	96%	23.20

reducing the HCP concentration by 7-fold. As a result Process Train 2A was actually capable of providing a slightly purer final product, although the difference is so slight that based on the accuracy of the HCP assay used, it is not possible to say that either process train had a higher purification capability than the other. The aim of testing the process sequence used for Process Train 2A, was to see whether the loss of product yield encountered during the diafiltration could be avoided by eliminating this step from the process. However the high level of product loss encountered over Capto S chromatography, used to facilitate the removal of the diafiltration step, meant that the overall process yield from Process Train 2A was actually only 12%, which is lower than the 31% obtained from Process Train 2. Based on the results from these two process trains, it would seem that the processing of a product pool containing high concentrations of PEG has serious implications upon the product recovery levels which may be achieved. Thus the feasibility of top phase processing must be called into question.

7.4.3 Process Train 3

In Process Train 3, Mabselect SuRe™ Protein A chromatography was used as the primary capture step, HPTFF was used for intermediate purification, whilst multi-modal chromatography using Capto adhere™ was used as the final polishing step. Compared to the integration of ATPE into Process Trains 2 and 2A, integration of HPTFF into Process Train 3 was relatively straightforward. The major consideration which needed to be accounted for was the low feed conductivity requirements for the HPTFF step. Since the HPTFF process utilised a membrane with a MWCO of 300kD which is larger than the IgG product, in order to maximise retention of the product,

it was necessary to fully exploit the positively charged nature of the membrane and the associated electrostatic forces it could exert. In order to do this, the conductivity of the feed and retentate needed to be maintained at a level of below 2.7mS/cm.[112] This ultimately required dilution of the Protein A eluate, post virus inactivation and neutralisation.

A sequential diafiltration strategy was employed for the HPTFF process in order to maximise the purification factor achievable. The diafiltration buffers used are summarised in Table 7.3

Table 7.3: HPTFF Diafiltration Buffers

Diafiltration Step	Buffer	pH	Conductivity (mS/cm)
1	10mM Sodium Acetate, 12mM NaCl	4.5	1.563
2	10mM Sodium Citrate	5.5	1.597
3	20mM Sodium Phosphate	6.5	1.485

The retentate was diafiltered with 10 diavolumes of each buffer. The use of such a large number of diavolumes may be considered excessive, as almost complete buffer exchange may be typically be accomplished using only 6-7 diavolumes (when using continuous diafiltration). However using a large number of diavolumes will also help to increase the purification factor of the process and the use of such volumes has been previously demonstrated.[112] In order to determine whether such large diafiltration volumes were actually necessary to achieve the desired level of impurity clearance, retentate samples were taken over the course of the HPTFF process and analysed for HCP content. The HPTFF process was performed using the same equipment set-up and methodology as was used for the initial evaluation experiments on the charged HPTFF membrane. The HPTFF process was operated at a feed cross flow rate of 323 LMH and a flux set point of 100 LMH.

Base Case 2

It should be noted that Process Train 3 utilised a new batch of feed material, obtained from different cell culture fermentation. Although the same cell line and cell culture process was used, the supernatant obtained from this culture had a lower titre than the previous batch of feed material used for the base case process, Process Train 2 and Process Train 2A. Furthermore, the impurity content was found to be slightly lower also. The HCP composition of the original cell culture supernatant was found to be approximately 16,000 ng/mg IgG, whereas in the case of the new feed material this figure was only 11,000 ng/mg IgG. In light of these differences, the base case process was run again, in order to provide a new process baseline against which the performance of Process Trains 3 and 4 could be compared. The results of this second base case run, using the new feed material is summarised in Table 7.4

Table 7.4: Summary of results from Process Train 2A

Step	Initial		Final		Yield	PF
	mAb Conc. (g/L)	HCP (ppm)	mAb Conc. (g/L)	HCP (ppm)		
Mabselect SuRe	1.31	10,966	3.57	51	79%	216.28
Capto adhere	2.72	49	2.54	7	96%	6.85
CaptoQ	1.92	<2	1.86	<2	100%	1.00

Comparing Tables 6.1 and 7.4 it can be seen that the change of feed material has impacted upon the performance of the base case process. The step yield on Mabselect SuRe has increased from 69% up to 79%, whilst the purification factor achieved on this unit operation has increased from 35-fold to 216-fold. The purification factor achieved across the Capto adhere step has also increased from 3-fold up to 7-fold. The implication of this is that direct comparison between the process trains which have used different starting feed materials is no longer valid. This however does not necessarily invalidate either set of results. Whilst direct juxtapositioning of these different data sets would not provide an accurate account of the relative performance of these process trains, it should still be possible to perform a certain degree of comparative analysis by normalising the results against the corresponding base case. Thus any following comparisons between the performance of Process Trains

2 and 2A to that of Process Trains 3 and 4 will be performed in this manner.

The change in feed material also had an impact upon the product quality. Purity analysis by SEC, revealed only monomeric IgG in the Capto Q product from the the first base case process. This however is not the case with the Capto Q product from the second base case process. The SEC result in Figure 7.9 shows that whilst the aggregate content is still low, there is now a small peak with a retention volume of approximately 3.1mL, which elutes from the SEC column after the monomer. There is also possibly another peak in the tail of the monomer peak with the retention volume of approximately 2.75mL. Since the Capto Q flowthrough contains only trace amounts of HCPs, these two peaks must be product related impurities and given that they elute from the SEC column after the monomer, this would suggest that they are either truncated antibodies, or fragments.

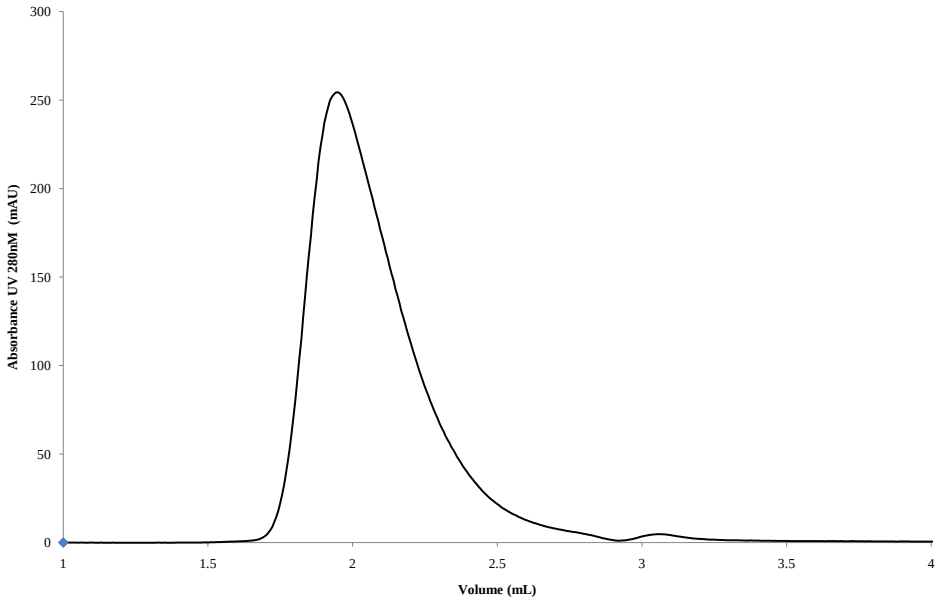


Figure 7.9: Chromatogram showing the purity by SEC of the product after Capto Q in the second base case run

Together, these fragments constitute approximately 2% of the total IgG (by peak area) present in the Capto Q product.

Process Train 3 - Mabselect SuRe Chromatography and Product Dilution

For Process Train 3, the Mabselect step was performed in the same manner as was done in the base case process. The Mabselect eluate was incubated at room temperature in order to achieve low pH viral inactivation, and then neutralised to pH 5.0 using 0.1M NaOH. The Mabselect step yield was calculated to be 76%, comparable to that which was achieved in the second base case process run, as was the purification factor of 162-fold. Figure 7.10 shows the purity of the Mabselect SuRe eluate as measured by SEC.

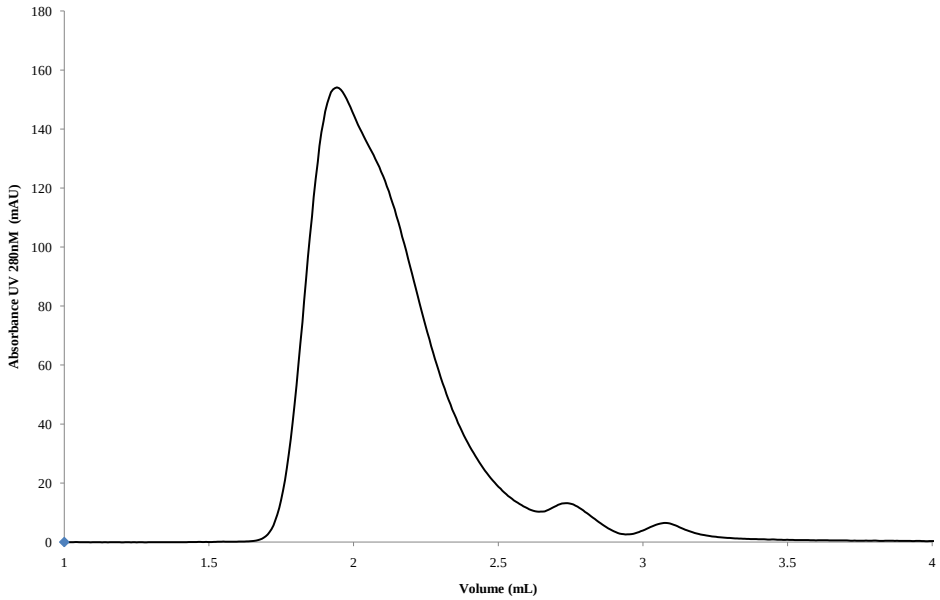


Figure 7.10: Chromatogram showing the purity by SEC of the product following Mabselect SuRe protein A chromatography, viral inactivation and neutralisation

As was seen in Figure 7.9, two peaks are present following the monomer peak on the SEC column. These peaks are most likely to be product related impurities, however their size in Figure 7.10 are significantly larger than they are in Figure 7.9. This would suggest that the Capto adhere and Capto Q steps in the base case process are capable of clearing these antibody fragments from the monomer product.

At this point, the conductivity of the neutralised Mabselect eluate pool was mea-

sured to be approximately 6.5mS/cm. It was felt that this conductivity was slightly too high for the HPTFF step to be effective, since the patents describing operation of the HPTFF process have the feed conductivity at levels of around only 2.7mS/cm.[112] As a result, the product pool was diluted 1 in 3 with MilliQ water, after which the conductivity was measured as 2.2mS/cm. Interestingly, dilution of the Mabselect eluate caused the HCP concentration to drop from 68 ppm down to 15 ppm. It could be the case, that the high level of dilution and the resultant low HCP concentrations may have reduced the accuracy of the ELISA assays used, leading to an underestimation of the actual HCP composition.

Process Train 3 - HPTFF

The HPTFF process was run in a sequential diafiltration mode. Initially the feed was concentrated approximately 10-fold in order to reduce the retentate volume, thereby reducing the diafiltration volume requirements and hence the associated process time. The retentate was then diafiltered with three different buffers at increasing pH. The purpose of this stepwise increase in diafiltration buffer pH was to maximise the purification factor achievable from the HPTFF step. Steadily increasing the system pH serves to reduce the net charge on any HCP components with a pI which is higher than the starting pH. As these components become less charged, their transmission through the positively charged HPTFF membrane should increase, whilst the product IgG, with its relatively basic pI is readily retained. Concomitantly, the purity of the IgG, in terms of total protein, should also increase.

Unfortunately the HPTFF step did not behave in the manner which it was expected to, with a high level of IgG transmission observed through the membrane into the permeate. During the initial concentration stage, approximately 10% of the product moved into the permeate. A further 27% was lost in the permeate during the first diafiltration stage, 26% was lost in the second diafiltration step and 15% was lost in the final diafiltration step. As a result the final overall step recovery from the HPTFF process was only 8%. It should be noted that volumes collected in the permeate during each of these steps were comparable.

The membrane used for the HPTFF step has a MWCO of 300kD which is larger than the product antibody. Retention of the target antibody is therefore reliant on the repulsive electrostatic forces between the positively charged membrane and the net positive charge carried by the antibody. The rationale for using a membrane with a MWCO greater than the size of the target antibody is that by doing so, it should be possible to cause components which have molecular weights comparable to that of the product to pass through the membrane into the permeate. This would therefore allow higher purification factors to be obtained, as was illustrated in the experiments used to evaluate the HPTFF membrane, in which a mixture of a polyclonal IgG and BSA were effectively separated using the technique. However the high level of product transmission through the membrane would suggest that the repulsive electrostatic forces being exerted by the membrane on the IgG were not sufficient to maintain the product in the retentate, even though experiments to evaluate the membrane using a polyclonal IgG suggested that it should be capable of this.

Analysis of the HCP composition of the retentate over the course of the HPTFF process revealed that the during the concentration stage, a 3.8-fold purification factor was achieved, with the retentate having a HCP content of 3.8 ppm. However the purity of the retentate actually decreased over the remaining diafiltration steps due to the high level of product transmission through the membrane. As a result of this, the final retentate purity could only be determine to be less than 10 ppm, due to the low IgG concentration in the product pool and the lower limit of detection for the HCP assays being reached. Based on this it would seem that it would have been better to operate the HPTFF step in Process Train 3 without the use of the sequential diafiltration steps as this would have resulted in the a process with a significantly higher recovery and also a reasonable level of HCP clearance.

Process Train 3 - Capto adhere Chromatography

For completeness the retentate recovered from the HPTFF process was passed through a Capto adhere step, using the same conditions as were previously employed. Due to the low IgG concentration of the feed material, the flowthrough from the Capto adhere

was not fractionated and analysed in order to determine the recovery and product purity at different points during the load and wash stages of the process, as was done in the previous Process Trains. Instead the flowthrough from the Capto adhere step was simply collected at UV280 absorbance values of greater than 50 mAU during the load and wash. Figure 7.11 shows the chromatogram obtained from the Capto adhere process.

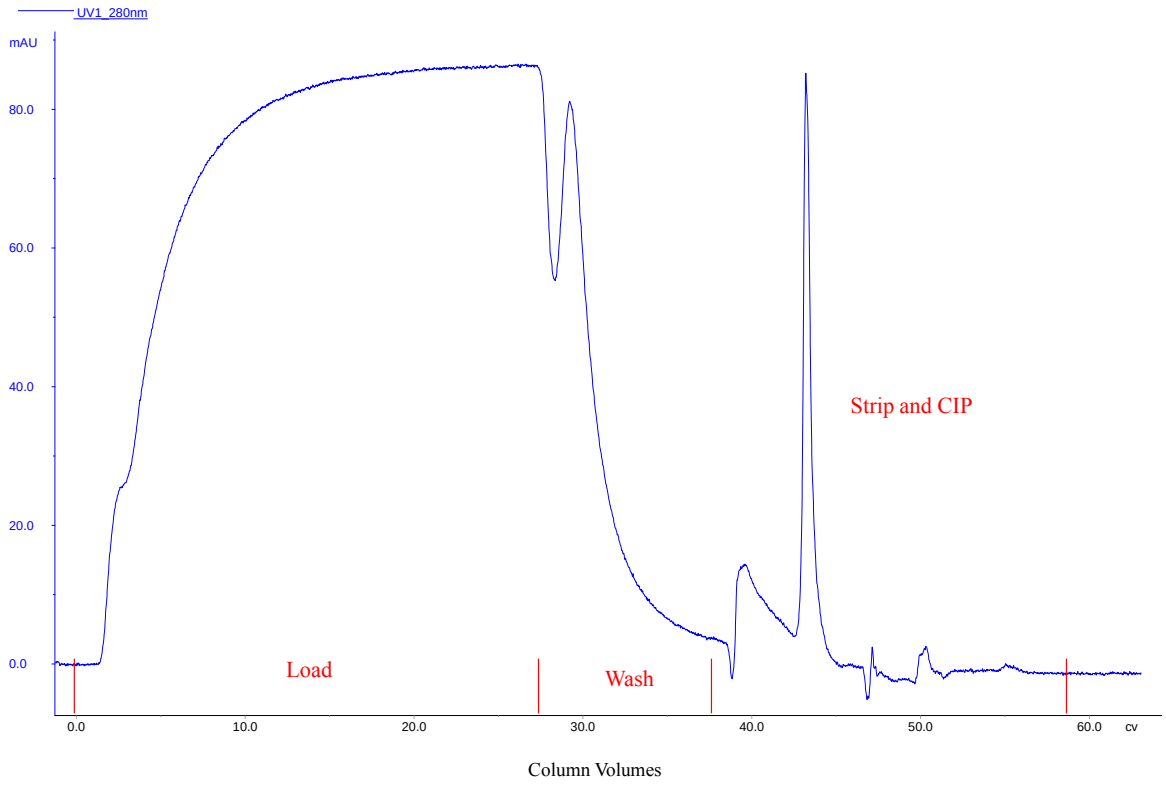


Figure 7.11: Chromatogram showing the UV absorbance at 280nm of the output from the Capto adhere step in Process Train 3

A step recovery of 84% was achieved from the Capto adhere process. The chromatogram shows some protein present in the strip and CIP fractions from the column. The Capto adhere step is known to bind some IgG. As described previously, increasing the IgG load on the Capto adhere column can help to reduce the resultant loss in yield. However given the low concentration of product present in the load material (the Capto adhere column was only loaded to a capacity of 12g/L resin), it is likely that the strip and CIP fractions contained the remaining 16% of product not accounted for in the collected flowthrough. The purification factor obtained from the

step was lower than one. However this is likely due to the HCP concentration being below the limit of detection. Instead, it is likely that the purity of the product loaded onto the column, is the same as that in the collected flowthrough, with impurities breaking through at the same time as the product IgG. The results obtained from Process Train 3 are summarised in Table 7.5

Table 7.5: Summary of results from Process Train 3

Step	Initial		Final		Yield	PF
	mAb Conc. (g/L)	HCP (ppm)	mAb Conc. (g/L)	HCP (ppm)		
Mabselect SuRe	1.24	10,964	3.61	68	76%	161.70
HPTFF	1.17	15	0.47	<10	8%	1.57
Capto adhere	0.47	<10	0.40	<12	84%	0.85

Figure 7.12 shows the results of the SEC analysis of the Capto adhere product.

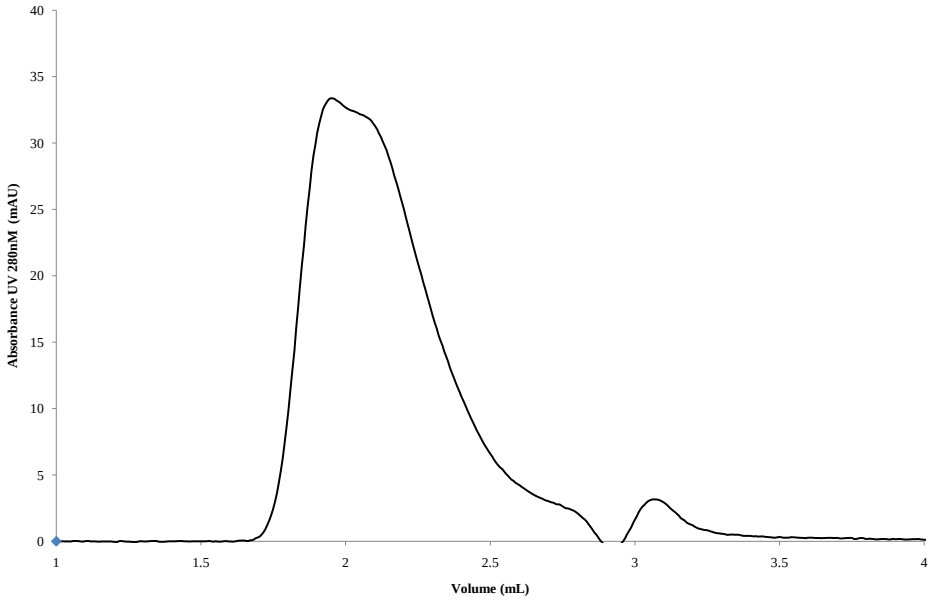


Figure 7.12: Chromatogram showing the purity by SEC of the Capto adhere flowthrough from Process Train 3

The results of the SEC analysis show that the fragmented IgG components present in the product pool following Mabselect capture, are still present in the Capto adhere flowthrough. The percentage of fragment has also decreased slightly from approxi-

mately 5% in the Mabselect eluate, to just over 3.5% in the Capto adhere flowthrough. It should also be noted that the fragment composition here is slight higher than it was in the product from the second base case process (1%) This would in turn suggest that Capto Q is responsible for the majority of the fragment clearance in the base case process, whereas HPTFF has been able to accomplish some clearance in Process Train 3. Since HPTFF is separating components based on size, it would be expected that the level of fragment removal would be relatively high. However it is likely that the high level of monomer transmission through the membrane has significantly compromised this ability.

The formation of truncated antibody molecules requires chemical bond cleavage. Given the types of chemical components used in this process, and their relatively moderate nature, it is unlikely that such cleavage could have been caused by the purification process itself. It is possible that protease enzymes, present in the cell culture supernatant may have been responsible for causing the formation of antibody fragments in the feed. Alternatively, the formation of these fragments may be as a result of some inherent instability, specific to this particular antibody product.

The chromatogram in Figure 7.12 also shows a slight double peak at a retention volume of approximately 2 mL. The first of these peaks is the monomer, based on previous SEC analysis of this product. However the component represented by the second peak is unknown. Whilst the chromatogram is Figure 7.10 did not show such a pronounced second peak, it can be seen that there was a slight shoulder on the tailing edge of the monomer peak. This was not present in second base case process (Figure 7.9). This second *monomer* peak could actually be another truncated form of the product which has formed in the feed material between the time in which the second base case and Process Train 3 were carried out. This is unlikely however, given that the feed materials were all frozen at -70^{o}C prior to use. An alternative explanation is the fact that the double peak is an artifact, formed by some degradation in the SEC column performance, and that both peaks actually correspond to the monomeric IgG.

Whilst aggregated forms of mAbs are known to be immunogenic, data on the immunogenicity of antibody fragments is not available. There presence in the product

pool is by no means desirable, since a reduction in monomer composition would ultimately result in a decrease in the active pharmaceutical ingredient (API) of the final formulated product. However their clearance is not an absolute requirement. Regardless, it would seem that the Process Train 3 is not capable of clearing as much of this product related impurity as the base case process.

Summary of Process Train 3

The results from Process Train 3 would suggest that it should be possible to integrate HPTFF into the three step base case process. Whilst the product recovery was severely compromised by the HPTFF step, analysis of the retentate following the initial concentration shows that it should be possible to achieve a sufficient level of HCP clearance without the use of the sequential diafiltration steps. Indeed, operating the HPTFF process in this manner would have resulted in a 80-90% step recovery (based on the product recovery in the permeate following the concentration step and accounting for potential product loss in recovering the IgG from the HPTFF system) and a product with a HCP composition of only 3.8 ppm. This could presumably be reduced to a level comparable to that of the final product from the second base case run, considering that in that process, Capto adhere was capable of providing a 7-fold purification factor. It would only need to provide a fraction of this performance in order to reduce the HCP composition by half. As it stands the overall yield from Process Train 3 was 5%. However if the diafiltration steps were removed from the process, then this yield could potentially be increased to over 54% (assuming an 85% step recovery from the HPTFF step). Furthermore, increasing the product yield and hence IgG concentration of the product pool following HPTFF would also potentially help to increase the yield on the subsequent Capto adhere step. There is therefore, clearly potential for integrating HPTFF into a three step purification process, however the level of product transmission through the membrane during the diafiltration steps is a cause for concern. In this case the purification factor required from the HPTFF process was fairly moderate, due to the high level of HCP clearance obtained from the Mabselect SuRe step. As a result the diafiltration steps which caused such high

levels of product loss could conceivably be eliminated from the process. However if the HPTFF process were required to clear more impurities than was the case in Process Train 3, then this low level of product retention would need to be addressed.

7.4.4 Process Train 4

The process sequence used for Process Train 4 was similiar to Process Train 3, with the only difference being that the Mabselect SuRe Protein A chromatography step was replaced by ATPE. The initial intention was to operate the ATPE step in the same manner as was used in Process Trains 2 and 2A, with the top phase from the back extraction recovered for further processing. Integration of this ATPE process into Process Train 4 proved to be problematic however due to the high conductivity of this top phase. As previously discussed, in order to ensure that the HPTFF step is operated effectively, the conductivity of the feed and retentate must be maintained at a sufficiently low level so as to ensure that the charged membrane can retain the product IgG. The results obtained from Process Train 3 would suggest that the conductivity of the feed material must be at least below 2.2 mS/cm.

Therefore one possible option for integrating these two processes, was to simply dilute the top phase from the back extraction to a sufficiently low conductivity and then use the HPTFF unit operation initially as a concentrating step, before diafiltering into the subsequent buffers for product purification.

However the conductivity of the top phase following the back extraction process was measured to be approximately 40mS/cm. As a result, such a strategy was felt to have been unfeasible since it would have required a top phase dilution of approximately 1 in 64 to bring the conductivity of the product pool to below 2.2mS/cm. An alternative approach was therefore proposed in which a diafiltration step was employed following ATPE and prior to HPTFF, using a standard membrane with a MWCO of 10kD. Such a strategy had previously been utilised in Process Train 2. However in that case a high level of product precipitation was observed leading to a low step recovery. As described previously, this precipitation was thought to have been caused by the high concentrations of PEG present in the feed material. Build

up of PEG near the membrane surface was felt to have potentially caused reductions in the local solubility of the product leading to the observed precipitation.

As a result of this, an alternative diafiltration strategy was used. The experiments used to develop the ATPE process had shown that it is possible to recover the product IgG in a bottom salt rich phase of a two phase system, by decreasing the amount of salt present in the forward extraction system, and using two sequential back extraction steps. In the first back extraction, the top phase from the forward extraction is contacted with a back extraction buffer forming a second two phase system, in which the product remains in the top polymer rich phase. In the second back extraction, the product enriched top phase from the first back extraction is contacted with more back extraction buffer to form a third two phase system, in which some product is recovered in the bottom salt rich phase.

The product recovery in this salt rich bottom phase was previously found to have been only approximately 55%. At the time at which this development work was performed, this level of recovery was felt to have been too low to consider taking forward. However considering the potential yield loss which would be encountered as a result of performing the diafiltration with the PEG enriched top phase from the first back extraction, coupled with the relatively modest yields achieved using Mabselect SuRe, it was felt that a recovery of only 55% might be acceptable under such circumstances. Scounting experiments utilising the two stage back extraction revealed that the product recovery in the bottom phase of the second back extraction step was actually between 65 and 70% using the new feed material (the aforemention yields of 55% were obtained using the previous feed material used for the first base case and Process Trains 2 and 2A). Whilst no further work was performed in order to characterise this behaviour, it could be speculated that differences in either the product concentration or the concentration of impurities between the two starting feed materials, has impacted upon the partitioning behaviour of the product IgG.

Process Train 4 - ATPE and Diafiltration

The ATPE process was performed in a similiar manner as was done in Process Train 2 and Process Train 2A. The two differences being that the NaCl composition of the forward extraction system was reduced from 12% (w/w) to 10% (w/w) and that an additional back extraction step was used to recover the product in a salt enriched bottom phase. The product recovery in the bottom phase of this second back extraction step was found to be 81%, which is actually highly comparable to the recovery achieved using Mabselect SuRe in the second base case process. One negative side-effect of using the multi-stage back extraction approach, as was alluded to previously when discussing the integration of ATPE into Process Train 2, is that a significant level of product dilution occurs as a result of the back extraction procedure. Indeed the process volume almost doubles, increasing by a factor of 1.6-fold, following ATPE. Whilst not an insurmountable problem, the increase in volume is not ideal, since the key objective for a product capture step, is to concentrate the product and decrease the process volume. The use of the diafiltration step immediately following ATPE helps to mitigate some of the concerns arising from this product dilution. Indeed, from a biomanufacturing perspective, if the diafiltration process is considered as part of the primary capture stage then the product dilution during back extraction is not really an issue at all, so long as there is sufficient tank space to hold the product pool during the diafiltration process.

Whilst the step yield from the ATPE process in Process Train 4 was comparable to that obtained in Process Train 2, the purification factors achieved were slightly different. In Process Train 2, the ATPE step was capable of a 6-fold purification factor, reducing the HCP concentration to 2,700 ppm. In Process Train 4, the ATPE step reduced the HCP concentration to 480 ppm. Although the starting HCP concentration was lower than was the case for the feed to Process Train 2, this still constituted a 23-fold reduction in HCP content over the ATPE step in Process Train 4. Thus it can be said that that change in feed material has had a beneficial effect upon the purifying power of the ATPE step.

The diafiltration process itself was also performed in the same manner as was done in Process Train 2, using a 10kD polyethersulfone membrane. The membrane was operated at a crossflow rate of 300L/m^2/h and a transmembrane pressure (TMP) of 1.5 bar. Prior to loading onto the diafiltration step, the bottom phase from the second back extraction was diluted 1 in 2 with MilliQ water, in order to reduce the viscosity of the feed, caused by the high salt concentrations of this bottom phase. It was hoped that this would help to increase the flux during the diafiltration process. Due to this dilution, prior to the actual diafiltration, the feed was concentrated by a factor of 22-fold. Further concentration was not performed due to constraints with the system hold-up volumes and therefore minimum working volumes. However even with the dilution, the membrane flux during this concentration step was found to initially be below 5 LMH. The total protein loading onto the diafiltration membrane was 377g/m^2. As a result of this, the time taken to perform the diafiltration step was very long, taking more than 12 hours. The speed of the process could be increased by decreasing the membrane load, however this would obviously come at the expense of increased cost of goods.

Following concentration, the product was initially diafiltered with eight diavolumes of 10mM Sodium Acetate, 150mM NaCl buffer at pH 4.5 and then with a further eight diavolumes of 10mM Sodium Acetate buffer and pH 4.5 which is the starting diafiltration buffer for the HPTFF step. The final membrane flux at the end of the diafiltration process was 25LMH and overall product recovery from this diafiltration step was 67%. Some precipitation was observed in the retentate, and it can only be assumed that some of this precipitate was product containing. Thus processing of the salt rich phase from the second back extraction did not completely eliminate the problems encountered during diafiltration in Process Train 2. However the recovery obtained is still significantly higher than that which was obtained in that previous process train. It should be noted that the final retentate IgG concentration was 1.3g/L, which is just over half the concentration reached during the diafiltration step in Process Train 2. It could therefore be argued that the level of precipitation is lower since the product concentration in the retentate is lower. Without further

concentrating the product in Process Train 4, it is not possible to determine whether this is indeed the case. However the IgG concentrations at this point are relatively low, and even in the presence of PEG, phosphate and citrate, it is unlikely that solubility limits are being reached.

Figure 7.13 show the SEC chromatogram of the product pool following diafiltration.

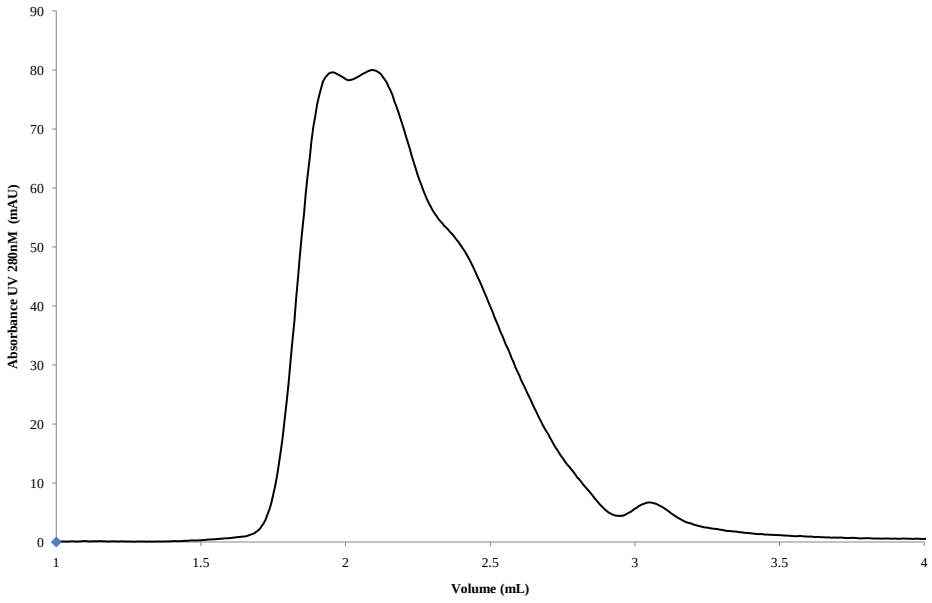


Figure 7.13: Chromatogram showing the purity by SEC of feed to the HPTFF Step in Process Train 4

As was seen previously with this batch of feed material, a peak corresponding to antibody fragments, with a retention volume of approximately 3 mL is present. Also as was seen when analysing the product samples from Process Train 3, there is a double monomer peak with a retention time of approximately 2 to 2.1 mL. In addition to this, the chromatogram shown in Figure 7.13 also shows an additional shoulder on the tailing edge of the monomer peak, with a retention volume of approximately 2.5mL. This peak could potentially correspond to a HCP component. It is highly dependent upon the UV280 absorbance characteristics of the protein in question, but given that the total HCP concentration at this point was approximately 1,500 ppm it is unlikely

that any single HCP could be responsible for the size of the peak/ shoulder seen in Figure 7.13. Instead it is more likely that the ATPE process has caused some form of product truncation. This antibody has been shown to have some inherent instability issues, as shown by the level of fragments present in the product pool from previous Process Trains. It is not inconceivable that the relatively harsh hydrophobic and ionic strength conditions to which the product is exposed to during the forward and back extraction stages of the process, may have enhanced these instabilities, leading to the formation of more fragmented IgGs. If this shoulder does indeed correspond to truncated antibody molecules, then fragments make up approximately 30% of the total IgG in the product pool.

Process Train 4 - HPTFF

The HPTFF process was performed in the same manner as was done in Process Train 3. The recovered product pool from the previous diafiltration step was diluted 1 in 2 with 10mM Sodium Acetate, pH 4.5 in order to increase the volume and aid in product handling. The diluted product pool had a conductivity of 0.47mS/cm. The feed was then concentrated until the minimum working volume for the HPTFF system had been reached. The same method of sequential diafiltration as was used in Process Train 3 was then employed.

As was found in Process Train 3, a significant amount of product transmission through the membrane was observed. During the initial concentration stage, approximately 6% of the product moved into the permeate. A further 50% was lost in the permeate during the first diafiltration stage, 45% was lost in the second diafiltration step and 20% was lost in the final diafiltration step. Based on these figures, the total yield of IgG in the permeate is greater than 100%. However there is likely to be some error in the measurements for the permeate fractions, since the concentrations were very low and towards the extreme end of the standard curve. It is therefore likely that the IgG concentrations of these permeate fractions were slightly overestimated. Regardless the level of product loss in the permeate was significant, and the final product recovery from the HPTFF retentate was only 3%.

The same yield loss was observed during the HPTFF process in Process Train 3, and as was the case then, the majority of product loss occurred during the sequential diafiltration stages. In Process Train 3, the recovery loss during the diafiltration stage of the process could be discounted to a certain extent since there was no increase in product purity during these steps. As a result, it would have been feasible to simply eliminate the diafiltration steps and operate the HPTFF just in concentration mode. A similiar approach cannot be adopted for the HPTFF step in Process Train 4, since the product pool following ATPE has a significantly higher HCP content. In Process Train 3, the feed for the HPTFF step had a HCP concentration of only 15 ppm. The concentration step of the HPTFF process was able to achieve a purification factor of 4-fold, reducing the HCP concentration to only 3.8ppm. In Process Train 4, the feed material had a HCP content of approximately 2300 ppm. The concentration stage was able to provide a 3-fold purification factor, decreasing the HCP concentration to 784 ppm.

Table 7.6 summarises the reduction in HCP concentration over the course of the HPTFF process run in Process Trains 3 and 4.

Table 7.6: Summary of HCP content of retentate over the course of the HPTFF processes in Processs Trains 3 and 4

	Process Train 3		Process Train 4	
HPTFF Stage	HCP (ppm)	PF*	HCP (ppm)	PF*
Load	15	-	2,265	-
Concentration	4	4	784	3
Diafiltration 1	8	0	351	2
Diafiltration 2	23	0	304	1
Diafiltration 3	8	3	740	0

* PF - Fold Purification Factor

From this it can be seen that the first two diafiltration stages of the HPTFF step in Process Train 4 did provide some benefit in terms of HCP reduction, whereas

in Process Train 3 there was none. The final diafiltration stage in Process Train 4 actually caused an increase in the relative amount of HCP but this is most likely attributable to the low concentration of product in the retentate at this point in the process, and limits of detection almost being reached on the analytical methods. Unlike in Process Train 3, the diafiltration stages are required to increase the purity of the product and as a result, they cannot be eliminated from the process. However the increase in purity afforded by these diafiltration steps, comes at the cost of a reduction in yield, and indeed the yield loss by the final diafiltration stage of the HPTFF process is so severe that even the product purity is compromised. It is apparent then that successful implementation of the HPTFF process, requires the membrane retention to be increased.

As described previously, it would seem that the electrostatic forces upon which the product retention is dependent, are not of a sufficient magnitude to maintain the product in the retentate. These electrostatic interactions between the membrane and the product could be enhanced by reducing the pH, in order to increase the net charge on the antibody. This was shown to have had an impact upon the retention of the polyclonal IgG during the early evaluation experiments performed on the HPTFF membranes. However the pH of the diafiltration buffers need to be slowly increased in order to reduce the net charge on positively charged HCPs, thereby facilitating their transmission through into the permeate. As a result, reducing the pH of the diafiltration buffers could possible come at the expense of reduced purification factors. An alternative approach would be to enhance membrane-product electrostatic interactions by decreasing the ionic strength of the buffers. This may not necessarily be feasible either since the conductivity of the diafiltration buffers used, did not exceed 1.6mS/cm, which must be considered fairly low. Further reductions in salt concentrations would not only reduce the buffering capacity of this diafiltration buffers, but may also compromise the stability in solution of the product. A final strategy would be to simply reduce the number of diafiltration volumes used. If it is assumed that the product flux (i.e. the rate of product loss into the permeate) remained constant during the course of each diafiltration stage, then reducing the number of diavolumes by

half, would also halve the yield loss. In this process 14 diavolumes were used for each diafiltration step, equating to approximately 2 complete buffer exchanges. Reducing this to 7 diavolumes and one complete buffer exchange would potentially decrease the purification factor since whilst product transmission is reduced, the level of impurities which move into the permeate would also decrease. Furthermore, halving the level of product loss during each diafiltration stage would only increase the product yield to approximately 30% which would not be a satisfactory level of recovery.

The low product retention is therefore a more fundamental problem than simply a case of using non-optimal operating conditions. Adjustments of pH, ionic strength and diafiltration volumes will only provide incremental improvements in product recovery, and these would most likely come at a high expense to the purification factors achievable. In order to increase the product retention it would be necessary to reduce the MWCO of the charged membrane. Rather than using a 300kD membrane, it would be necessary to reduce the nominal pore size to perhaps 100kD. This would increase product retention, but would also increase the retention of some impurities, so once again the increased recovery would come at the expense of reduced purification factors.

The 300kD charged HPTFF membrane was shown to effectively retain the polyclonal IgG used during the initial evaluation experiments. Figure 7.14 shows a comparison of the chromatograms obtained from SEC analysis of both the polyclonal IgG used for the evaluation experiments and the monoclonal IgG used for these whole process sequence experiments.

The polyclonal IgG SEC chromatogram shows that there are two discreet groups of IgGs with comparable sizes, while as expected the monoclonal IgG has only one. Figure 7.14 also shows, based on the retention volumes that the monoclonal IgG is smaller than the smallest group of IgG present in the polyclonal IgG. The difference in molecular size of these two components may help to explain the lower than expected retention of the monoclonal IgG, however the level of difference in retention is surprising. IgG molecules generally have molecular sizes ranging from approximately 100 kD to 150 kD. Even if it is assumed that the polyclonal IgG molecules are at

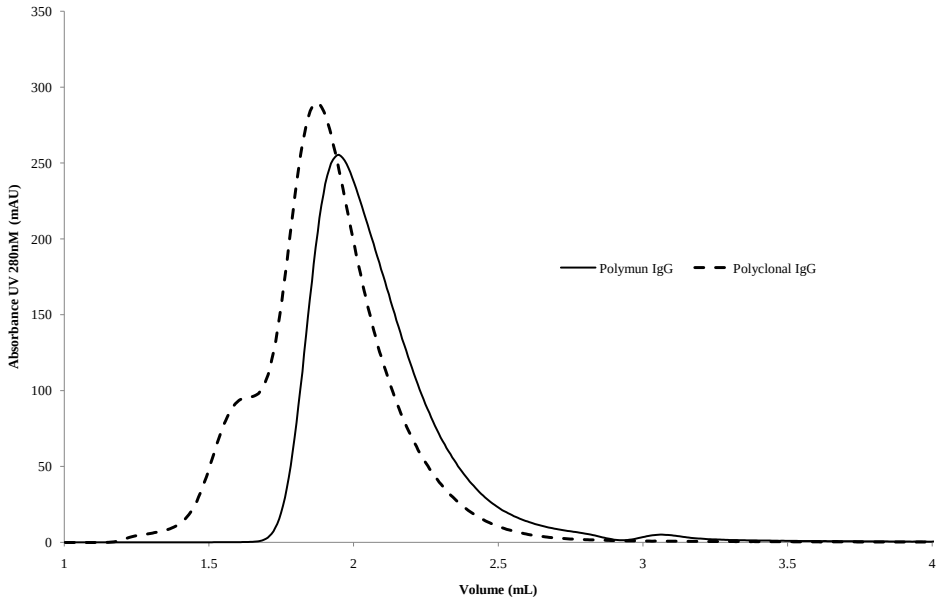


Figure 7.14: Comparison of SEC chromatograms of the monoclonal antibody used for these process integration experimental studies, and of the polyclonal IgG used for the initial evaluation experiments on the charged HPTFF membrane.

the top end of this range, and that the monoclonal IgG is at the bottom, this is still a fairly small size range across which a fairly dramatic change in membrane retention has occurred. Therefore it must be assumed that the charge characteristics of the monoclonal antibody are significantly different to those of the polyclonal IgGs. Indeed, the partitioning of the IgG in the ATPE system would suggest that the monoclonal antibody has hydrophobic tendencies, implying a lack of charged groups on the protein surface. If this is indeed the case, then this would help explain why the positively charged membrane was unable to exert electrostatic repulsive forces of a sufficient magnitude in order to maintain the product in the retentate. It would also explain, as was previously alluded to, the reason why Capto S displayed such poor binding capacities in Process Train 2A. Regardless, the results obtained point to potential issues with the *platforming* of HPTFF processes, since the differences in the performance of the charged HPTFF membrane between the two different antibody products (polyclonal and monoclonal), show that the product retention performance is highly sensitive to variations in the size and charge characteristics of the product. Whilst some sensitivity may have been expected, since after all the HPTFF affects bioseparation by exploiting differences in these particular molecular characteristics, the level of sensitivity significantly compromises the likelihood of adopting a platform approach to HPTFF process development.

It is clear from these results that the MWCO of the charged membrane will need to be reduced in order to increase the retention of the product. Thus it would seem that membrane pore sizes would need to be selected on a case by case basis depending on the charge and size characteristics of the product. Comparing this to chromatography processes, selecting membrane pore sizes is similiar to selecting chromatography resins. The difference however is that in a platform process, the chromatography resin is typically fixed, and it is simply the operating parameters such as bed dimensions and mobile phase characteristics which are altered. This is not the case with HPTFF it would seem, where membrane pore size would need to be optimised along with the operating conditions for each new product.

One point which has not been addressed is the state of the membrane following

these HPTFF experiments. One further possible explanation for the poor retention of IgG, is that the charged ligands have somehow been stripped from the membrane, thereby reducing the charge density on the membrane surface. Such an outcome is unlikely. Water flux test showed that the NWP was consistent at the level observed during the evaluation experiments described in Chapter 6. As was observed then, the immobilisation of these charged ligands to the membrane, reduced its effective pore size. As a result, loss of ligands would most likely be accompanied by an increase in the NWP, which was not observed. Also, loss of ligands would require the cleavage of chemical bonds. The physicochemical conditions to which the membrane was exposed during these experiments were likely not harsh enough to cause such effects. To ensure that the membrane had not lost any charged ligands, it would have been necessary to repeat the IgG-BSA studies which were performed previously to characterise the membrane. Comparison of the results would then have allowed any degradation in membrane performance to be observed. This experiment was not performed.

Process Train 4 - Capto adhere

As was the case for the Process Train 3, for completeness the recovered retentate from the HPTFF process was loaded onto a Captoadhere column. Due to the low concentration of the load material, the flowthrough was not fractionated, and was instead simply collected at UV280 absorbances of greater than 5 mAU. The chromatogram for the Capto adhere step is shown in Figure 7.15

The Capto adhere step was able to provide a 3-fold purification of the IgG (in terms of total protein), and a step yield of 77%. From Figure 7.15 it can be seen that several peaks were seen in the strip and CIP steps. The first two of these peaks were also present in the Process Train 3 Capto adhere chromatogram. Given that the load material onto the Capto adhere step in Process Train 3 was mainly composed of IgG, with only trace amounts of HCP present, these first two peaks are most likely anitbody being stripped from the column. Thus the 23% of product unaccounted for in the collected flowthrough is most likely present in these strip peaks. As was the case in Process Train 3, the yield loss on Capto adhere can most likely be reduced

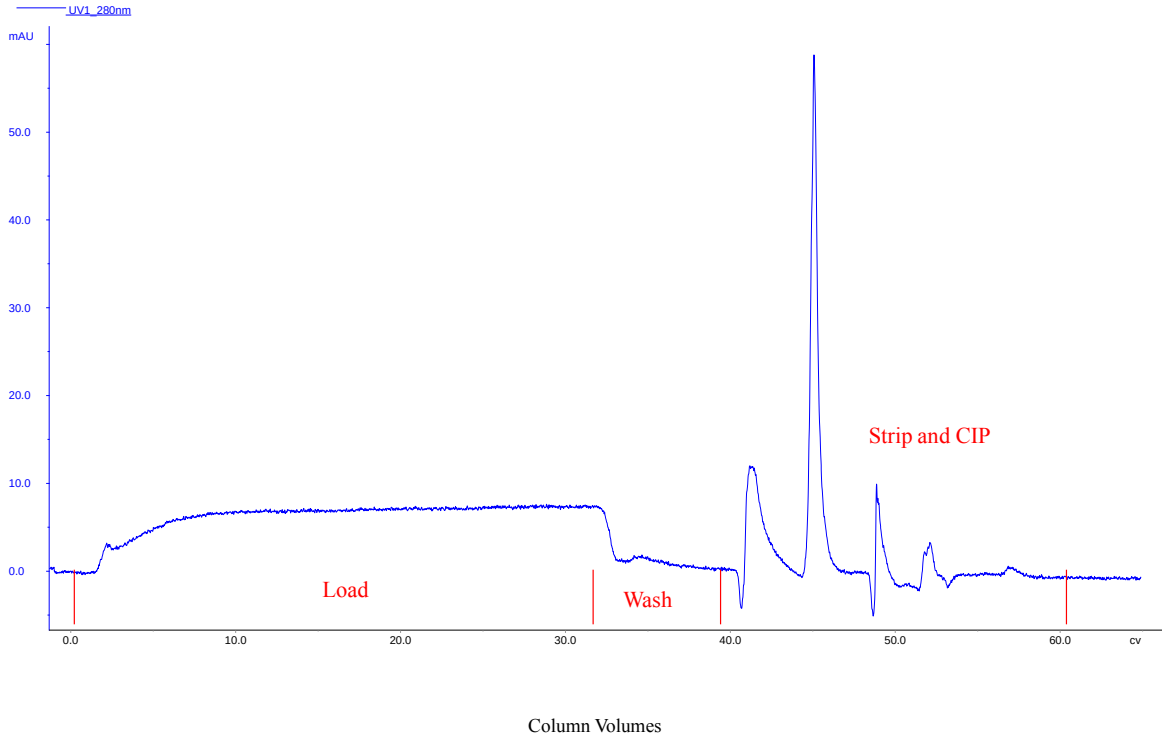


Figure 7.15: Chromatogram showing the UV absorbance at 280nm of the output from the Capto adhere step in Process Train 4

by increasing the load on the column. In this case the load capacity was only 1.6g/L resin.

Table 7.7 summarises the result obtained from Process Train 4.

Table 7.7: Summary of results from Process Train 4

Step	Initial		Final		Yield	PF
	mAb Conc. (g/L)	HCP (ppm)	mAb Conc. (g/L)	HCP (ppm)		
ATPE	1.24	11,113	0.62	479	81%	23.20
Diafiltration	0.29	886	1.29	1,582	67%	0.56
HPTFF	0.54	1,481	0.05	740	3%	2.00
Capto adhere	0.05	740	0.04	250	77%	2.96

Summary

The overall process yield and purity of the final product from Process Train 4 were of an unacceptably low level. There were however some encouraging results obtained. Firstly it was possible to recover the product IgG in the salt rich bottom phase during ATPE, with a step recovery comparable to that which had been achieved using Mab-select SuRe Protein A chromatography. This is something which had not previously been accomplished using the first batch of feed material, during the development of the ATPE process. The diafiltration step also provided a significantly higher recovery than had previously been observed in Process Train 2. In this regard the ATPE and diafiltration steps, effectively representing the primary capture portion of the process train, functioned fairly well. The only major drawback is the relatively low resolving power provided by the ATPE process. Whereas Protein A chromatography was capable of reducing the HCP concentration from 11,000 ppm to 50 ppm, ATPE was only able to offer a fraction of this purification factor, bringing the HCP concentration from 11,000 ppm down to approximately 500 ppm.

The change in the partitioning behaviour of the product and hence the recovery of IgG in the salt rich phase of the second back extraction system, when using the new batch of feed material, provides some insight as to the relative robustness of the ATPE process. Process development work on the ATPE process indicated that the ATPE system developed by Andrews et al.[110] was not behaving in the manner in

which it was expected, thereby revealing that differences in performance should be expected between different products. The fact that the system behaviour also deviates significantly when using different batches of the same product further emphasises the lack of robustness inherent to this ATPE system (recalling that the feed material for the ATPE processes used in Process Train 2 and Process Train 4 were essentially the same). The major difference is the product concentration and hence the concentration of other process related impurities. Such variations could be expected in upstream processes, with titres varying from batch to batch. If the partitioning behaviour of the ATPE system is sensitive to such variations, then it suitability for primary capture purposes is highly limited. It could be argued that the Protein A chromatography also displayed a certain lack of robustness between the two feed batches, although these differences were not as significant as in the case of the ATPE process.

As was the case in Process Train 3, the performance of the HPTFF step was inadequate, providing only a low level of purification and a high level of product loss. The majority of this loss of product occurred during the diafiltration stages of the process, similiar to what occurred during the HPTFF step in Process Train 3. However due to the low purity of the starting feed material, it cannot be argued that the diafiltration steps can be sacrificed in order to increase the process yield since this would almost certainly compromise the purification power of the HPTFF process. It would therefore seem that integration of ATPE and HPTFF in the manner in which they were for Process Train 4 requires further development of the HPTFF step in order to boost the purification factor achievable from the overall process. As it stands however, the performance of Process Train 4 falls some way short of that of the base case process.

7.4.5 Process Train 5

In Process Train 2, ATPE was used to replace the Mabselect SuRe Protein A capture step. This was then followed by diafiltration, Capto adhere chromatography and then finally Capto Q. The overall performance of that particular process train was not capable of matching the performance of the base case process. Whilst the final

product had a HCP concentration of just over 100 ppm, which could be considered reasonable, particularly for an unoptimised process, the overall process yield was very low at approximately 30%. This low recovery was predominantly caused by the low yield obtained from the diafiltration process following ATPE, used to buffer exchange the product pool in order to reduce its conductivity, thereby allowing it to be loaded onto the Capto adhere step.

This loss of yield was thought to have been caused by the presence of large amounts of PEG in the feed, a side-effect of being unable to achieve high product recoveries in the bottom salt rich phase during the ATPE back extractions. In Process Train 4 however, as a result of switching to a new batch of feed material, it was found that it was possible to acheive high recoveries of the product in a salt rich phase, by utilising two batch extraction steps. Furthermore the level of product loss during the subsequent diafiltration step was significantly reduced, presumably as a result of decreasing the PEG present in the system.

In light of this result, the process sequence making up Process Train 2 was re-evaluated using the two stage back extraction procedure for the ATPE step, in order to see if the overall process recovery could be improved, thereby making the ATPE - Capto adhere- Capto Q process sequence more viable as an manufacturing option. This new process sequence was labelled Process Train 5

ATPE and Diafiltration

The ATPE and diafiltration steps were performed in the exact same way as they were carried out in Process Train 4. Following diafiltration, the recovered retentate was diluted with a 10% (v/v) shot of 250mM Na-Phosphate pH 7.0 and then titrated to pH 6.5 using 0.1M NaOH. The product pool was then loaded onto the Capto adhere step. The results, in terms of recoveries and HCP composition of the product pool, from the ATPE and diafiltration step of Process Train 5 were the same as those obtained in Process Train 4.

Capto adhere and Capto Q

The Capto adhere and Capto Q steps were performed in the same manner as they were in the base case process. The Capto adhere column was loaded with the titrated product pool to a concentration of approximately 42g/L resin. In order to maximise the step yield, the flowthrough from the Capto adhere step was collected at absorbance values greater than 30 mAU. The chromatogram for the process is shown in Figure 7.16.

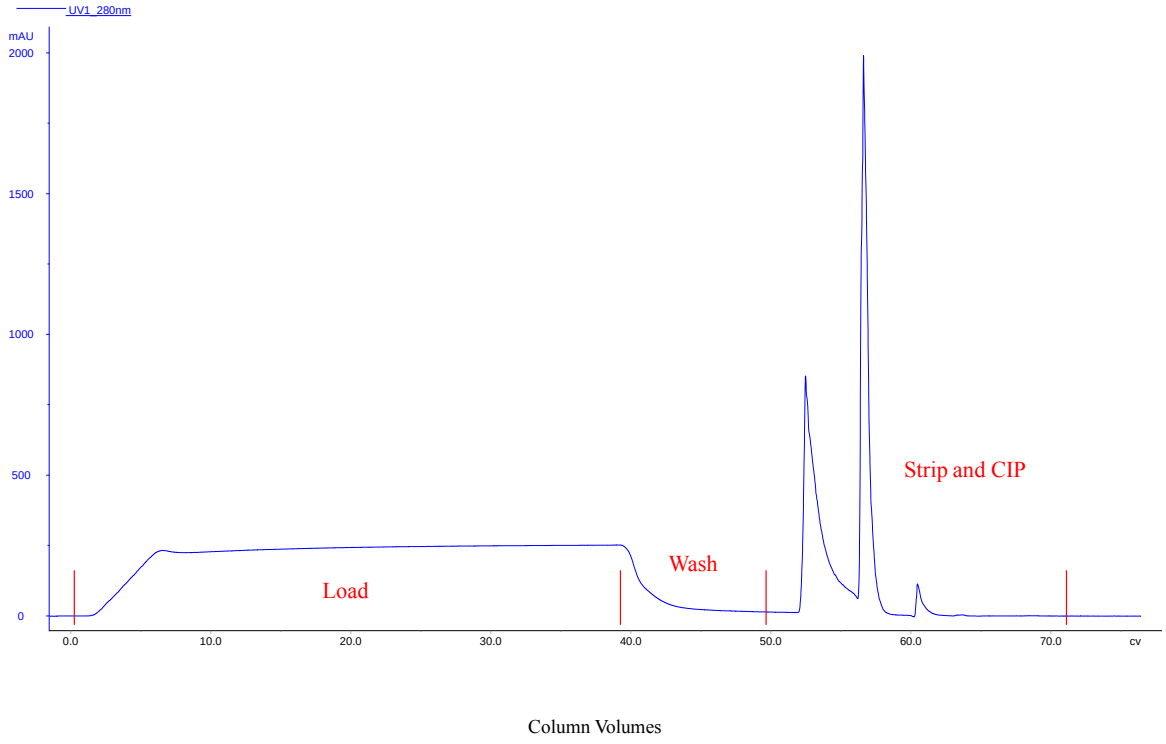


Figure 7.16: Chromatogram showing the UV absorbance at 280nm of the output from the Capto adhere step in Process Train 5

The Capto adhere step was able to provide an approximate 9-fold purification factor, which is over four times greater than the 2-fold purification factor achieved using the same step in Process Train 2. The step yield was 98%. Following Capto adhere, the product pool was buffer exchanged into 25mM Na-Phosphate pH 7.5 using a desalting column, after which it was loaded onto a CaptoQ column which provided as step yield of 97% and a purification factor of just over 1, which is lower than the

7-fold purification factor obtained across the Capto Q step in Process Train 2. The product pool following the Capto Q step had a HCP concentration of 118 ppm which is comparable to the 109 ppm present in the Capto Q flowthrough from Process Train 2. Thus overall, even though the ATPE process was capable of providing a higher level of impurity clearance, the final product still contains the same concentration of HCP.

Figure 7.17 shows the results of the SEC analysis of the Capto Q product.

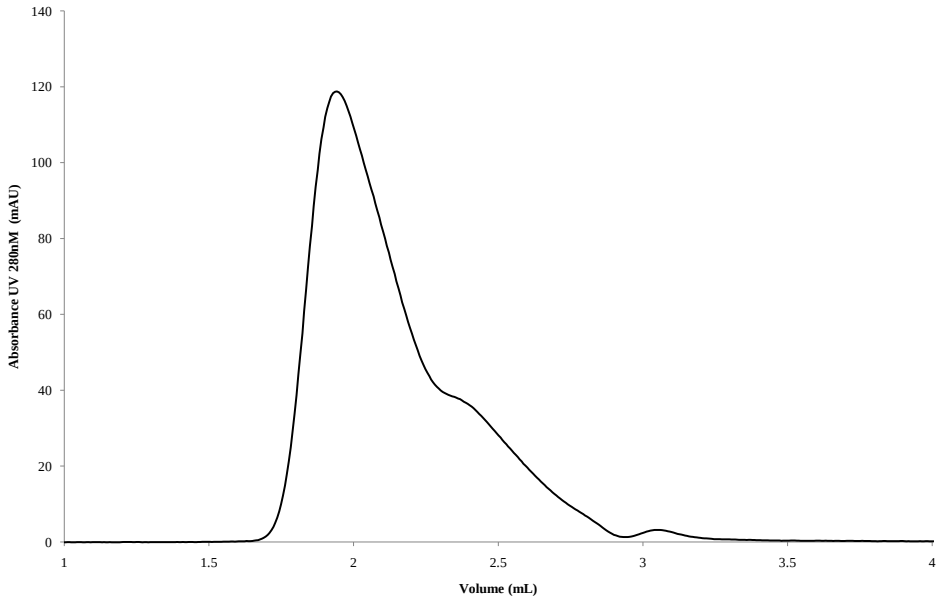


Figure 7.17: Chromatogram showing the purity by SEC of the Capto adhere flowthrough from Process Train 5

The SEC analysis of the product pool following diafiltration in Process Train 4 showed that, as well as the two fragment components with retention volumes of approximately 2.75 and 3.1 mL, the use of ATPE and diafiltration also possibly caused the formation of a third fragment species, appearing as a shoulder on the tailing edge of the monomer peak with a retention volume of approximately 2.3mL. From this analysis, it was determined that antibody fragments made up approximately 30% of the IgG present in the product pool. The SEC trace shown in Figure 7.17 shows that this third antibody fragment is still present in the product pool following Capto

Q. However the size of the shoulder corresponding to this fragment is now smaller, relative to the main monomer peak. Based on peak areas, these fragments now only make up 20% of the IgG present in the Capto Q flowthrough, indicating that either Capto adhere or Capto Q has helped clear some fragment from the process. Based on data from previous process trains, it is most likely to be the latter.

Table 7.8 summarises the results from Process Train 5.

Table 7.8: Summary of results from Process Train 5

	Initial		Final			
Step	mAb Conc. (g/L)	HCP (ppm)	mAb Conc. (g/L)	HCP (ppm)	Yield	PF
ATPE	1.24	11,113	0.62	479	81%	23.20
Diafiltration	0.29	886	1.29	1,582	67%	0.56
Capto adhere	1.10	1,756	0.99	177	98%	9.82
CaptoQ	0.80	135	0.76	118	97%	1.14

Summary

The results from Process Train 5 show that by altering the ATPE back extraction process, so that product could be recovered in a salt enriched bottom phase the overall process yield could be improved over that which was obtained from Process Train 2, in which the product was recovered in a polymer rich top phase. This increase in process yield is almost entirely attributable to the increase in the diafiltration step recovery. However, even though the overall process yield was improved, the final product was of a comparable purity (in terms of HCP content) to that of the Capto Q product from Process Train 2, with the final HCP concentration at about 100 ppm in both cases. The way in which HCPs were cleared from the process stream differed slightly however. In Process Train 2, the diafiltration step provided a significant level of HCP clearane, with a 8.4-fold decrease in HCP concentration achieved across this step, whilst the HCP clearance provided by the Capto adhere step was minimal In Process Train 5, the diafiltration step did not result in any reduction in the HCP concentration. Instead the ATPE step provided a 23-fold reduction in HCP (compared to the 6-fold reduction in Process Train 2), whilst the Capto adhere step provided a nearly 9-fold reduction in HCP concentration. The Capto Q provided only a neglible

level of HCP clearance unlike in Process Train 2 where it was capable of reducing the HCP concentration by over 7-fold. The difference in the purification performance of the purification steps is most likely due to a combination of the change in the feed material and also the alteration made to the back extraction method. Comparison of the performance of the platform process run for the two base case processes, shows that the change in the batch of feed material used impacted upon the behaviour of the unit operations present in these process trains. As a result the increased level of HCP clearance observed during the ATPE and Capto adhere steps may be partially attributable to the change in the HCP composition of the feed material. The fact that the product was recovered in the salt rich phase of the back extraction system may also have contributed to the change in the HCP clearance behaviour of the process. The partitioning behaviour of components in an ATPE system is dependent upon the characteristics of the component molecules. By changing the phase in which the product is being recovered, the type of impurities which are being co-purified with the product will also change. Thus the Capto adhere and Capto Q steps in Process Train 5 are most likely being challenged with an entirely different set of HCPs than they were face with in Process Train 2, which may further explain the change in purification factors obtained. The results from the SEC analysis show that ATPE and diafiltration are possibly responsible for the formation of an additional form of truncated antibody, which is not present in the feed material. Whilst the remaining steps in the process are capable of reducing the amount of this fragmented form of antibody in the product pool, they are not capable of removing it all. The exact cause of this fragmentation and the means through which it may be mitigated is beyond the remit of this particular study. However based on the fact that fragments were already present in the feed material, it is likely that this particular antibody, or at least this batch of material has some inherent instability issues associated with it. The conditions to which the product is then exposed to during the ATPE and diafiltration processes then serve to exacerbate these instabilities leading to the observed fragmentation. Overall the results from Process Train 5, show that it may be technically feasible to use an ATPE process as a primary capture step, integrated into the platform process in place of

Protein A. The overall process yield is 20% lower than the base case, and the HCP concentration is approximately 10-fold higher, however considering the non-optimised nature of Process Train 5, these results should be considered fairly encouraging. The major concerns at this point would be the antibody fragmentation observed and also the fact that an additional diafiltration step is required in order to integrate the ATPE process into the platform.

7.5 Conclusions

The aim of this study was to evaluate the performance of ATPE and HPTFF, the two most industrially attractive alternative bioseparation techniques identified by the MADM based framework described in Chapter 5, when used as part of a whole downstream purification process.

The primary conclusion which may be drawn, based on the results obtained, is that none of the process trains incorporating the use an alternative bioseparation technique is capable of matching the performance of the standard Protein A based platform process. In this case performance is measured based on metrics of overall process yield, HCP clearance and product purity (in terms monomer composition). The protein A platform process out performs all of the alternative containing process trains on each of these counts. However the aim of this study was not principally to determine whether any of these alternative could outperform the platform process. Indeed it could be argued that based on the metrics of performance used, and considering that neither ATPE or HPTFF were optimised for this particular separation, it would be almost impossible for the alternative containing process trains to outperform the Protein A platform process. Instead the aim was to determine how these techniques performed when used as part of a whole process train and indeed how *close* they could come to matching the performance of the base case platform process. The isolated nature in which the alternatives were evaluated in the MADM based analysis detailed in Chapter 5, it could be argued, was unfair to many of these alternative techniques as it did not account for how these techniques performed from a whole process perspective.

The results from this study have gone some way towards addressing these concerns, as data is now available on whole process train performance.

Another aim of the experiments performed in this study was to generate relevant experimental data regarding the performance of these techniques when used for the purposes of antibody purification. A further criticism which could be levelled at the MADM based analysis detailed in Chapter 5 is that the data used to model these alternative techniques was based on available literature values, which often times did not actually involve the purification of mAb products. The process performance data collected from this study can therefore be used in a "second-pass" analysis of ATPE and HPTFF using the MADM based framework, in order to assess the industrial attractiveness of these alternatives, using correctly contextualised data.

A third, and in retrospect, key aim of this study was to provide hands-on experience with the operation of these techniques such that a better understanding of the ease of which these techniques may be incorporated into an actual purification process, and how they may actually be utilised for large scale biomanufacturing, could be obtained. The collective results obtained from the studies detailed in this chapter and those in Chapter 6 have helped this aim to be met. Subtleties regarding the use of ATPE and HPTFF in the context of a whole purification process, which were not immediately apparent from the literature which was review, have now been revealed. In particular issues of process integration and the compatibility of these techniques with other unit operations within the platform process have been effectively uncovered.

The prime example of this is ATPE. A number of different methods were utilised in order to integrate the ATPE process into the purification train, including the use of diafiltration and the replacement of the multi-modal intermediate purification step with standard cation exchange chromatography operating in bind and elute mode. In all cases, yield loss was encountered as a result of either poor binding to the cation exchange column, or as a result of product precipitation during the diafiltration. The level of product loss was worst when trying to process the top polymer rich phase of the back extraction system, and the optimal performance was obtained when recovering the product in the bottom phase and using a diafiltration step in order to reduce

the ionic strength of the process stream, making it compatible with the next step in the process train. The incompatibility of the ATPE product with the remaining steps in the purification train, and the need for an additional buffer exchange step to integrate ATPE into the platform process actually compromises the high throughput advantages which may be associated with this particular alternative. Thus whilst the ATPE step itself may be relatively quick to perform compared to Protein A packed bed chromatography, any increase in productivity is most likely negated by the relatively low throughput of the diafiltration step. Taking this into account, a more suitable approach may have been to use a unit operation which is not sensitive to ionic strength conditions such as hydrophobic interaction chromatography, for the intermediate purification step. Such an action would have however constituted a change to the platform which was beyond the remit of this study.

Whilst issues were also encountered in integrating HPTFF into the platform process, such as the need for product pool dilution in order to reduce process stream conductivities, these were not as severe as was the case with ATPE. Instead the major issue encountered with the use of HPTFF was the unexpectedly low product retention displayed by the HPTFF membrane. The early evaluation experiments described in Chapter 6 gave no indication that product retention would be an issue. However the results of these whole process experiments would show that even with products of the same modality (i.e. IgGs), slight differences in either the molecular size or charge can have dramatic impacts upon the process yields which may be attained. Such sensitivities would then call into question the feasibility of "platforming" the HPTFF process, such that the same operating conditions, and in particular the same membrane type, could be used across a range of different antibody products. Given the results which have been obtained, this is unlikely to be possible, and it will be necessary to optimise the membrane MWCO for each product.

As stated previously the overall performance of the process trains which incorporated the use of either ATPE, HPTFF or both, were not able to match that of the Protein A base case platform process, in terms of product purity or yield. However the differences in performance were not sufficiently large so as to completely rule out

the feasibility of adopting these techniques for the purification of antibody products. This is particularly true, when it is considered that neither ATPE of HPTFF were truly optimised for the purification of this particular antibody product.

A potential criticism of the MADM based framework described in Chapter 5 is that the alternative techniques were evaluated in isolation, rather than as part of a whole process sequence. It could be argued that such an analysis is unfair since techniques were penalised for not providing a sufficient level of performance in terms of, for example, the purification factor which might be achieved. From the perspective of a bioprocess engineer, purification factor is a key process parameter, however it is a parameter which is associated with the whole process, rather than with any one individual step. Thus even if ATPE is unable to provide sufficient impurity clearance compared to Protein A chromatography, this does not matter if the remaining steps in the purification train can effectively "pick up the slack". The results of the whole process sequence experiments performed in this study show that this may indeed be the case. The process train in which ATPE was used to replaced Protein A chromatography, provided an overall process yield which was 80% of the yield achieved using the base case platform process and the final product had a HCP concentration of only 100 ppm. Whilst this may be high compared to the 2 ppm present in the final product from the base case process, it is still acceptable considering the unoptimised nature of not only the ATPE step, but also the subsequent chromatography steps, which were not developed for use following a ATPE capture process.

Indeed whilst it could be argued that neither the ATPE nor the HPTFF systems used, performed as well as was expected, it could be also be argued that such shortcoming could be overcome through additional process development effort. In the case of ATPE, this would most likely involve the use of high throughput screening methods to obtain conditions which provided more favourable product partitioning behaviour. In the case of HPTFF, the evaluation experiments have already shown the potential of this technique for protein separation, the membrane pore size simply needs to be adjusted in order to improve product retention during the process. Thus, from a very high level perspective it would seem that it would be feasible, at least from a tech-

nical standpoint, to incorporate ATPE and HPTFF into a mAb purification process. Whilst incorporating both of these techniques together does not provide sufficient performance, the results of the experiments show that either swapping out Protein A for ATPE or using HPTFF for intermediate purification does provide decent outcomes. There are however a number of caveats. Firstly, the use of either ATPE of HPTFF reduces the feasibility of a platform process in which only minor operating parameters need to be altered between different products. Both these alternative techniques have been shown to be sensitive to changes in the product stream characteristics, requiring major changes to be made to the process in order to make them work. In the case of HPTFF, the membrane pore size cannot be fixed and must be adjusted based on the size and charge characteristics of the antibody molecule. ATPE also displayed significantly different behaviour to that which was described by the original developers of the system, implying that a blank sheet approach to development would likely need to be taken for each new ATPE process, utilising high throughput screening methods to determine optimal operating parameters, including the type of polymer-salt system to use.

Another caveat is that to this point no account has been made of matters related to process economy. Whilst the performance, in terms of yield and purification power, of these alternatives was always unlikely to match that of their packed bed chromatography counterparts, one area in which they may potentially have an edge is in terms of the associated cost of goods and also the levels of productivity which they may provide. Indeed it is in these areas in which the concerns surround packed bed chromatography are based, and it is the primary reason why techniques such as ATPE and HPTFF have been proposed as suitable replacements, with the potential to provide higher process throughputs at a reduced cost. Thus whilst the results presented in this chapter show that it is at least technically feasible to incorporate ATPE and HPTFF into a mAb purification process, the question now becomes whether it is worth incorporating them from an process economy perspective.

In the following chapter, details will be provided of how the results obtained from this study were used to generate basic process economy models for each of the

bioseparation techniques which were used. These models were then used to determine the cost of goods associated with each of the process trains used in this study, as well as the overall process productivity. By comparing the cost and productivity of each of these process trains to that of the base case platform process, it was hoped that the question of whether the incorporation of these alternative techniques into the mAb purification process is a worthwhile endeavour, could be successfully addressed.

Chapter 8

Process Economy Evaluation of Alternative Techniques

8.1 Abstract

In this chapter a study was undertaken to study the potential process economy advantages which may be conferred through the adoption of alternative bioseparation techniques, from a whole process perspective. Process models were generated to simulate the performance of the process trains examined in Chapter 7. These were used to calculate a number of process economy related parameters, such as productivity and cost of goods (CoGs) for each of the process trains. Comparison of the different process trains in terms of these process economy metrics allowed the advantage of utilising ATPE and HPTFF to be discerned. The results of the analysis showed that whilst the use of alternatives did indeed result in improvements in the productivity and/or CoGs of these processes, the improvements obtained were not sufficiently significant. In the case of ATPE, improvements in productivity were severely compromised by the need for protracted steps, required to integrate this technique in to the purification process. HPTFF meanwhile only provided sufficient benefits with the major caveat that the process performed as it was expected rather than as it was observed during the experimental studies. The results therefore indicate that the use of process economy as justification for adopting these alternative bioseparation techniques into mAb

purification techniques is not sufficient and cannot be used to revoke the performance and robustness advantages conferred by the Protein A platform process.

8.2 Introduction

Chapters 6 and 7 provided details of experimental studies performed to assess the performance of ATPE and HPTFF when used as part of a three-step mAb purification process. The aim of these studies, was to determine how the techniques behaved when used as part of a whole purification train, and also how the performance of these process trains compared to that of a base case platform process, made up of a Protein A primary capture step, a multi-modal chromatography intermediate purification step and finally a cation exchange polishing step. The aim was to address any concerns surrounding the fact that the MADM based framework described in Chapter 5 analysed these alternative techniques in isolation rather than as part of a whole process, and as a result could have lead to an underestimation of their true potential. Incorporated into this aim were a number of additional goals, including the generation of data reflecting the performance of these techniques when used specifically for mAb purification and also to gain a general impression of the ease in which these techniques may be developed and operated.

The results of these experimental studies showed that none of the process trains which incorporated the use of ATPE and/or HPTFF, were able to match the performance, in terms of yield and HCP clearance, of the base case process. However considering that the ATPE and HPTFF processes used were never optimised for this particular separation, it was unlikely that this would ever have been the case. Instead the major insight which may be taken from the experimental studies presented in Chapters 6 and 7, is that whilst the performance of these "alternative" process trains fell short of matching that of the base case process, the differences observed were not sufficient so as to completely rule out the feasibility of using ATPE in place of Protein A, for primary capture and HPTFF as an intermediate purification step of a mAb product. Indeed whilst the ATPE and HPTFF systems did not perform as well

as possibly expected, it could be argued that such shortcoming could be overcome through additional process development effort.

If therefore it is technically feasible to incorporate these techniques into a mAb purification process, the key question then becomes whether their incorporation is worthwhile. The answer to such a question must factor in a number of different considerations. Firstly there is the issue of process economy. As detailed in Chapter 2 the initiative to find alternatives to packed bed chromatography, for the purification of antibody products, is driven by two process economy related components. There is the relatively high cost associations, particularly with Protein A chromatography and there is also the potential for chromatography to impose a capacity, or productivity constraint upon a manufacturing facility. One of the attractive features of many of the proposed "alternative" bioseparation techniques is the potential benefits which they may provide in terms of these key, process economy related aspects. ATPE for example is a bulk separation technique, and therefore has the potential to scale with the process volume rather than, as is the case with Protein A chromatography, the mass of product which needs to be captured. As a result, ATPE could potentially overcome the capacity and productivity constraints which may be associated with packed bed chromatography. HPTFF is a membrane based separation technique and as a result does not rely on the use of costly chromatography resins. This potentially makes HPTFF significantly cheaper to operate than packed bed chromatography.

The counterpoint argument to this is the fact that the use of a Protein A based platform process, presents benefits beyond just high process performance (in terms of yield and impurity clearance). It also provides process robustness which in turn has several knock-on effects. The first of which is that it vastly simplifies process development. The use of a process template means that process development scientists have a scaffold upon which to develop the purification process. This constrains the number of process parameters that need to be optimised which in turn increases the speed and reduces the resource requirements associated with process development. This process robustness also simplifies manufacturing, making the concept of multi-product facilities much more feasible, since the same process will essentially be used

each time. Considering the large investments of time and money required to retrofit facilities to house new processes, or even to build entirely new facilities from scratch, this is a significant benefit. And it is a benefit which is unlikely to be shared by process trains incorporating the use of alternative bioseparation techniques.

Based on these points, it therefore comes down to a question of exactly how much of a benefit in terms of productivity and cost savings, if any, does the utilisation of these alternative bioseparation techniques provide, and whether or not these benefits are of a sufficient magnitude to justify the abandoning of a platform approach to mAb purification. The process trains incorporating the use of alternative bioseparation techniques, have however, to this point only been evaluated in terms of performance related metrics such as yield and purification power. The advantages of productivity and reduced cost of goods which they may present have not yet been addressed.

In light of this, a study was initiated in order to quantitatively assess the cost and productivity advantages provided by the use of ATPE and HPTFF. Using the data obtained from the experimental studies detailed in Chapter 7, process models were generated and used to determine the cost of goods and process productivity associated with each of the process trains which were investigated, and also the individual unit operation of which they were comprised. By using the base case process as a bench mark, the aim was to quantitatively determine the exact magnitude of the cost and productivity benefits inferred by the use of these alternative techniques.

8.3 Materials and Methods

The analysis of the process economy metrics for each of the process trains, was performed with the aid of mass balance models generated using Microsoft Excel. These models were based on those which were used for MADM analysis described in Chapter 5. In order to ensure a fair comparison could be performed, each of the process trains were modelled to process a single batch of clarified cell culture supernatant from a 20,000L fermenter, expressing mAb at a titre of 5g/L, approximately reflecting the maximum IgG concentration encountered during the experimental studies detailed in

Chapters 6 and 7. The large cell culture volume of 20,000L was chosen in order to fully reflect the potential productivity advantages inferred through the use of alternative bioseparation techniques, and concomitantly, to uncover any cost and capacity issues which might be associated with Protein A packed bed chromatography.

8.3.1 Process Models

Each of the unit operations used in the process trains tested in Chapter 7 were modelled individually. These were then combined to form whole process models representing the process trains. The key required outputs from the process models were the batch operating costs, and the batch time for each of the process trains . These could then be used to calculate the associated process productivity and CoGs. To this end, the models incorporated the necessary equipment, throughput and materials requirements of each of the unit operations comprising the process trains being analysed. Whilst the values for the key operating parameters used for each process train model were based on the corresponding experimental results which were obtained, a number of assumptions were made for each of the unit operations.

Chromatography

All of the chromatography processes were modelled as single cycle operations. Columns were sized based on the capacity observed during the experimental studies and the feed volume. The column diameter was constrained at a value of 200cm, reflecting the largest sized chromatography columns which are currently available. The bed height was then calculated based on this, and the bed volume requirement. In cases in which the required bed height was less than 10cm, the column diameter was decreased to 100cm.

The maximum superficial fluid velocity through each chromatography column was set at 300cm/hr, in order to reflect the decrease in bed stability which is commonly observed when scaling up the use of compressible chromatographic resins. In most cases, this superficial velocity resulted in residence times which exceeded those used during the experimental studies. This was done to ensure that the capacities observed

would be maintained. In situations in which this minimum residence time was not met, the superficial fluid velocity was decreased accordingly.

All chromatography resins were assumed to have a lifetime of 200 cycles. The buffer volumes required for each step of the chromatography processes, were based on those used in the experimental studies.

Ultrafiltration

The buffer exchange operations performed between the multi-modal and anion exchange chromatography steps of the base case process and Process Trains 2 and 5, were modelled as ultrafiltration/ diafiltration processes. In the experimental studies, this step was achieved using a size exclusion column, however scaling of such a process to handle a 20,000L batch is not feasible. For the ultrafiltration/ diafiltration step an average flux of 50 LMH was assumed for the duration of the process. A total protein load of 600 g/m^2 was used to determine the required membrane area. The ultrafiltration processes were modelled to utilise seven diavolumes in order to ensure complete buffer exchange. The membrane area, flux and process volumes were then used to calculate the total process time. The ultrafiltration steps following ATPE in process trains 2 and 5 were modelled in the same manner.

ATPE and HPTFF

Each of the phase forming steps (forward and back extractions) of the ATPE process were modelled as being carried out in stirred tanks. The phase separation steps were modelled as centrifugation processes. Other process parameters such as phase ratios and process yields were based on those observed during the experimental studies. The recycling and reuse of polymers were not modelled.

HPTFF was modelled as essentially an ultrafiltration/ diafiltration process. A membrane loading of 600 g/m^2 was again used, although this was higher than that which was utilised during the experimental studies. The flux through the membrane was set at 100LMH. Again yields from the HPTFF process were set to values observed during the small scale experimental runs. The cost of the HPTFF membranes were

set at double those of standard ultrafiltration membranes, to account for the added electrostatic charge.

8.3.2 Process Economy Metrics

The process economy metrics required for this analysis, were the productivities and CoGs of the process trains as well as the unit operations of which they were comprised.

Productivity

The productivity was calculated based on the total amount of product processed and the process time. The amount of product processed was in turn based upon the amount of product present in the feed material and the process yield. Productivity was then calculated by dividing this quantity by the process time as shown in Equation 8.1

$$\text{Productivity} = \frac{\text{Amount of IgG in Feed} * \text{Process Yield}}{\text{Process Time}} \tag{8.1}$$

These productivity calculations were all performed on a per batch basis. Thus productivity was expressed as the batch productivity with units of grams per hour (g/hr).

Cost of Goods (CoGs)

The CoGs associated with the process trains and each of the unit operations from which they were composed, was calculated using cost components of capital expenditure, raw materials costs and consumables costs, and were again based on a per batch basis. The capital expenditure was basically made up of the cost of all the equipment required to perform each process step. These costs were in turn based upon the same cost database and models as were used in the MADM analysis described in Chapter 5. The batch capital expenditure was calculated by spreading this cost across the total number of batches which could be completed using this equipment as described by Equation 8.2

$$\text{Batch Capital Expenditure} = \frac{\text{Total Capital Cost}}{\text{Equipment Lifetime}} \tag{8.2}$$

The "equipment lifetime" parameter was determined as shown in equation 8.3, based on the assumption that 140 batches could be performed in one year, and that the plant had an operating lifetime of ten years, over which the equipment would depreciate to a value of zero. Thus the batch capital expenditure was calculated by dividing the cost of the necessary equipment by a figure of 1,400.

$$\text{Equipment Lifetime} = \text{No. of Batches Process Annually} * \text{Plant Operating Lifetime} \tag{8.3}$$

The raw materials and consumables costs were calculated based on the quantities of these components required for a single batch.

With regards to raw materials, a general assumption made was that all process buffers, with the exception of those used for the ATPE process, had a cost of \$2 per litre. The same cost was not used for ATPE buffers as these all had higher salt concentrations than would typically be encountered in bioprocessing. As a result ATPE buffer costs were calculated based on the cost of the individual buffer components.

The total batch cost, for each unit operation and also the total process train, was then determined through the summation of each of these individual cost components.

The CoGs was calculated by dividing this batch cost, by the amount of material processed as shown in Equation 8.4.

$$\text{CoGs} = \frac{\text{Total Batch Cost}}{\text{Amount of Product in Feed} * \text{Process Yield}} \tag{8.4}$$

Table 8.1 summarises all the assumptions that were made in the modelling of these different process trains.

Table 8.1: Key assumptions for the process models used for process economy calculations

Process Step	Process Parameter	Assumption
Protein A Chromatography	Max Bed Height	20 cm
	Max Bed Diameter	200 cm
	Dynamic Binding Capacity	30 g/L
	Linear Fluid Velocity	300 cm/h
	Resin Lifetime	200 Cycles
	Resin Cost	\$10,000 per litre
	Elution Volume	3.97 CVs
Multi-Modal Chromatography	Max Bed Height	20 cm
	Max Bed Diameter	200 cm
	Dynamic Binding Capacity	100 g/L & 179 g/L
	Linear Fluid Velocity	300 cm/h
	Resin Lifetime	200 Cycles
	Resin Cost	\$4,000 per litre
	Flowthrough Dilution Factor	1.1
AEX & CEX Chromatography	Max Bed Height	20 cm
	Max Bed Diameter	200 cm
	Dynamic Binding Capacity	100 g/L & 142 g/L
	Linear Fluid Velocity	300 cm/h
	Resin Lifetime	200 Cycles
	Resin Cost	\$2,000 per litre
	Flowthrough Dilution Factor	1.1
Ultrafiltration/Diafiltration	Membrane Load	600 g/m^2
	Average Flux	50 LMH
	Diafiltration Volumes	7
	Membrane Lifetime	200 cycles
	Membrane Cost	\$1,250 per m^2
ATPE	Forward Extraction Phase Ratio	0.81
	Back Extraction 1 Phase Ratio	1.22
	Back Extraction 2 Phase Ratio	0.04
	PEG Cost	\$40 per kg
	Phosphate/ Citrate Cost	\$30 per kg
HPTFF	Membrane Load	600 g/m^2
	Average Flux	50 LMH
	Diafiltration Volumes	7
	Membrane Lifetime	200 cycles
	Membrane Cost	\$2,500 per m^2

8.4 Results and Discussions

8.4.1 Summary of Process Performance

The yield achieved by the process trains tested in the experimental studies detailed in Chapter 7 are summarised in Table 8.2. The process train numbering has been maintained from that used in Chapter 7. The exception is Process Train 1.1, which refers to the second base case process which was run, using the new batch of feed material.

Table 8.2: Summary of yields achieved by individual unit operations and overall process trains tested during experimental studies

	Process Trains						
Step	Train 1	Train 2	Train 2A	Train 1.1	Train 3	Train 4	Train 5
1	69% (PA)	86% (ATPE)	86% (ATPE)	79% (PA)	79% (PA)	81% (ATPE)	81% (ATPE)
2	-	41% (DF)	-	-	-	67% (DF)	67% (DF)
3	96% (MM)	98% (MM)	15% (CEX)	96% (MM)	8% (HPTFF)	3% (HPTFF)	98% (MM)
4	95% (DF)	95% (DF)	95% (DF)	95% (DF)	-	-	95% (DF)
5	105% (AEX)	97% (AEX)	96% (AEX)	98% (AEX)	84% (MM)	77% (MM)	97% (AEX)
Overall	66%	32%	12%	71%	5%	1%	49%

*P*A - Protein A, MM - Multi Modal, CEX - Cation Exchange, AEX - Anion Exchange, DF - Ultra/Diafiltration

The overall process yields achieved using the process trains which incorporated the used of ATPE and/or HPTFF were all lower than those of the platform processes (Process Trains 1 and 1.1). Process trains which involved the use of HPTFF all had very low process yields which, as was discussed previously in Chapter 7, were mainly attributable to the unexpected poor product retention characteristics of the charged HPTFF membrane.

Process trains 2, 2A and 5 all utilised ATPE for primary capture in place of Protein A chromatography. Process trains 2 and 2A whilst not displaying as poor yields as those achieved from the process trains which incorporated the use of HPTFF, were still fairly low. In the case of Process Train 2 this was attributable to the product

loss observed due to precipitation during the diafiltration step following ATPE. In the case of Process Train 2A, the poor yield was a result of poor product binding to the cation exchange column.

Of all the process trains which included the use of an alternative bioseparation technique, Process Train 5, which utilised ATPE for primary capture, displayed the highest overall process yield. A overall process yield which was approximately 70% of that achieved using the base case process was attained. This may be considered a fairly decent result considering the non-optimised nature of the process.

8.4.2 Process Productivity

As previously described, a major potential advantage possessed by ATPE and HPTFF are the relatively high process productivities, compared to packed bed chromatography, which may be achieved using these techniques. In order to assess the magnitude of this advantage, the process models were used to determine the productivities of the process trains tested during the experimental studies described in Chapter 7. Table 8.3 summarises the productivities of each of the process trains tested during the experimental study, as well as the productivities of the individual unit operations of which they are comprised.

Table 8.3: Summary of productivity achieved by individual unit operations and overall process trains tested during experimental studies

	Process Train Productivity						
Step	Train 1	Train 2	Train 2A	Train 1.1	Train 3	Train 4	Train 5
1	1.00 (PA)	7.32 (ATPE)	7.32 (ATPE)	1.00 (PA)	1.00 (PA)	4.04 (ATPE)	4.04 (ATPE)
2	-	0.64 (DF)	-	-	-	0.44 (DF)	0.44 (DF)
3	2.05 (MM)	2.42 (MM)	0.04 (CEX)	1.92 (MM)	0.19 (HPTFF)	0.06 (HPTFF)	2.54 (MM)
4	2.45 (DF)	1.02 (DF)	0.26 (DF)	2.80 (DF)	-	-	2.26 (DF)
5	3.27 (AEX)	1.27 (AEX)	0.47 (AEX)	2.87 (AEX)	0.08 (MM)	0.03 (MM)	2.43 (AEX)
Overall	1.00	0.51	0.06	1.00	0.07	0.02	0.57

*P*A - Protein A, MM - Multi Modal, CEX - Cation Exchange, AEX - Anion Exchange, DF - Ultra/Diafiltration

Productivity values were initially expressed in grams per hour (g/hr), and were calculated by dividing the mass of IgG in the product stream by the time taken to complete the process. In order to facilitate comparison of process productivities, the values presented in Table 8.3 for the individual unit operations have been normalised against the productivity displayed by the Protein A step of the corresponding base case process. This was to account for the fact that two different base case processes were run, each using a different batch of feed material. Thus the productivity of unit operations in Process Trains 2 and 2A have been normalised with respect to the productivity displayed by the Protein A step in Process Train 1, whilst unit operations in Process Trains 3, 4 and 5 have been normalised against the productivity of the Protein A step in Process Train 1.1. The overall process productivities meanwhile have simply been normalised against the corresponding base case. Thus the productivity of Process Trains 2 and 2A have been normalised against the productivity of Process Train 1, whilst the productivity of Process Trains 3, 4 and 5 have been normalised against that of Process Train 1.1.

From the data presented in Table 8.3, in the base case process, the Protein A step has the lowest productivity, with the remaining unit operations in the process having an approximately 2 to 3-fold greater throughput. This is mainly a result of the other chromatography steps in the base case platform process, being operated in flow-through, rather than bind and elute mode.

In terms of alternatives, the benefit provided by the use of ATPE is immediately apparent, at least in terms of the process productivity. ATPE provides a 4 to 7-fold increase in productivity over Protein A chromatography. HPTFF meanwhile fares less well since the productivity is severely compromised by the low process yield exhibited during the experimental studies.

Process Trains 2, 2A and 5 all utilised ATPE for primary capture. Of these, the ATPE process in Process Train 2 and 2A displays the highest productivity, at approximately 7 times that of the Protein A step. This is attributable to the fact that in Process Train 2 and 2A, only a single back extraction step is performed, with the top phase from this step passed on for further processing. In Process Train 5, a second

back extraction step is utilised in order to recover the product in the bottom phase. It is this additional step which causes the observed decrease in productivity. Despite the high productivities displayed by ATPE, the remaining steps in the downstream process have generally lower productivities that their corresponding steps in the base case process, resulting in the overall process productivities for these process trains being lower than that of the platform process.

For example, Process Train 5 has an overall process productivity which is only approximately 60% that of the base case process (Process Train 1.1). The data in Table 8.3 shows that the multi-modal chromatography step in Process Train 5 has a higher productivity than that of the same process in Process Train 1.1. This is primarily due to the higher product concentration in the Capto adhere feed in Process Train 5, as it was assumed that the ultrafiltration step could be used to concentrate the feed. The Capto Q anion exchange step in Process Train 5 meanwhile has a lower productivity than its counterpart in Process Train 1.1, which is attributable to the lower assumed binding capacity, set to account for the higher HCP and impurity profile of the load material. However, whilst this makes some contribution, it is not predominantly responsible for the relatively low overall process productivity of Process Train 5. A significant difference between these two process trains is the presence of the ultrafiltration step following ATPE, which is not present in the base case platform process. Table 8.3 shows that this step actually has a lower productivity than Protein A, and it is this which causes the observed low overall process productivity. Indeed if the model parameters are altered such that the productivity of the multi-modal and anion exchange chromatography steps, as well as the ultrafiltration process between them in Process Train 5, match that of Process Train 1.1, the overall productivity of Process Train 5 is still only approximately 70% of that of the base case.

It can be seen therefore that whilst ATPE does provide a significant benefit in terms of process productivity, the difficulties encountered in integrating this alternative technique into the platform process, have resulted in a relatively low overall process productivity.

8.4.3 Cost of Goods

Aside from improvements in productivity and throughput, another potential advantage over packed bed chromatography, offered by these alternative techniques is the reduced cost of goods (CoGs). This is essentially the cost of manufacturing a unit mass of product and can be expressed in terms of dollars per gram ($/g). As with the productivity, absolute values for the CoGs associated with each process train and the individual unit operation of which they were comprised, were initially calculated using the generated mass balance models. These values were then normalised against the CoGs associated with the Protein A step of the corresponding base case process. Table 8.4 summarises the CoGs for each of the process trains tested during the experimental study.

Table 8.4: Summary of cost of goods (CoGs) for individual unit operations and overall process trains tested during experimental studies

	Process Train CoGs						
Step	Train 1	Train 2	Train 2A	Train 1.1	Train 3	Train 4	Train 5
1	1.00 (PA)	0.69 (ATPE)	0.69 (ATPE)	1.00 (PA)	1.00 (PA)	0.93 (ATPE)	0.93 (ATPE)
2	-	0.66 (DF)	-	-	-	0.46 (DF)	0.46 (DF)
3	0.17 (MM)	0.27 (MM)	15.86 (CEX)	0.19 (MM)	6.08 (HPTFF)	16.23 (HPTFF)	0.26 (MM)
4	0.27 (DF)	0.33 (DF)	0.48 (DF)	0.27 (DF)	-	-	0.24 (DF)
5	0.18 (AEX)	0.26 (AEX)	0.32 (AEX)	0.21 (AEX)	0.44 (MM)	1.24 (MM)	0.25 (AEX)
Overall	1.00	2.12	14.05	1.00	12.58	23.69	1.58

*P*A - Protein A, MM - Multi Modal, CEX - Cation Exchange, AEX - Anion Exchange, DF - Ultra/Diafiltration

In terms of the base case, as expected, Protein A has the highest CoGs associated with it, contributing over 60% of the total processing costs per batch. However comparing Protein A to the ATPE steps used for primary capture, it can be seen that the CoGs values calculated are actually fairly comparable. The ATPE system used in Process Trains 2 and 2A has a CoGs value which is approximately 30% lower than that of Protein A, whilst the ATPE system used in Process Trains 4 and 5 have CoGs which are only 7% lower.

The cause of this is predominantly down to raw materials. Whilst ATPE does not utilise any expensive consumables (such as Protein A chromatography resin), it does make use of vast amounts of polymer and salt, to form the requisite two phase systems. These raw materials cannot be re-used (the possibility or recycling the polymer was not considered in this case). As a result of this, the batch processing costs for an ATPE and Protein A chromatography process are very comparable. In the case of Process Train 5, the batch cost is actually less that 5% lower than that of Protein A in the base case, which is reflected in the highly comparable CoGs.

The equipment costs associated with the ATPE processes were also approximately 50% higher than for Protein A, due to the need for multiple mixing tanks and centrifuges for phase separation. The equipment requirements for Protein A chromatography are comparatively modest. The equipment costs were accounted for in the cost of goods, by assuming a batch schedule of 140 batches per year and an equipment lifetime of ten years. The equipment costs were then spread across this lifetime in order to determine the costs associated with a single batch. Due to the manner in which the equipment costs were accounted for, the actual contribution to the CoGs was minimal. The difference, or similarities in this case, in the CoGs between Protein A and ATPE is therefore almost entirely attributable to the raw materials and consumables costs associated with each technique.

The overall CoGs associated with process trains incorporating the use of HPTFF are expectedly high due to the poor yields attained using the HPTFF process. The difference in the CoGs of the HPTFF steps in Process Trains 3 and 4 is due to the poorer yield obtained from this step in Train 4. The CoGs associated with the cation exchange chromatography step in Process Train 2A is also very high, again due to the poor step yield obtained using this process.

Of all the process trains tested, only Trains 2 and 5 have comparable overall CoGs values to the base case process, although even these are at least 50% greater than that of the platform process. Process Trains 2 and 5 are essentially the same as the platform process, the difference being that the Protein A capture step has been replaced by an ATPE process. Process Train 5 differs from Process Train 2 in that the ATPE step

utilises an additional back extraction step, which not only decreases the productivity, as was described previously, but also increases the cost of goods. As a result the CoGs associated with the ATPE step in Process Train 5 is approximately 15% greater than that in Process Train 2. Regardless, whilst they are comparable to the base case, the overall CoGs are lower in both instances. This is primarily due to the presence of the additional ultrafiltration/ diafiltration step required to condition the product pool to enable its loading onto the following multi-modal chromatography step. The CoGs associated with these steps are high in both cases. Indeed with Process Train 2, the CoGs of this diafiltration step is almost the same as that of the preceding ATPE process. The reason for this is the relatively modest yield achieved across this step as a result of product precipitation. In the case of Process Train 2, the diafiltration yield was particularly bad at approximately 40%. In Process Train 5, the yield on this step was partially improved to approximately 70%. In any case, the inclusion of this additional process step resulted in an increase in the overall CoGs.

Based on these results, it would therefore seem that the use of alternative bioseparation techniques has not had an beneficial effect upon the CoGs of the process. In the case of HPTFF, the poor process yields achieved during the experimental studies resulted in very high CoGs, whilst in the case of ATPE, the vast amounts of raw materials consumed by the process means that even though the process does not utilise any consumables, the overall operating costs are still comparable to those associated with Protein A packed bed chromatography. The CoGs of process trains incorporating the use of ATPE are actually increased by the need for intermediate conditioning steps, in a similar manner to the way productivity was decreased by them. This data would therefore suggest that there is at least no advantage in using either ATPE or HPTFF in terms of CoGs.

8.4.4 Specific Productivity

The productivity of these process trains have been previously analysed. There is however an alternative way of expressing the productivity of a process, accounting for the cost of achieving a particular throughput. For example, the productivity of a

ultrafiltration process may be improved by reducing the membrane load and increasing the membrane area. In this regard the improvement in productivity has come at a price. In order to account for this, the productivity of the process may be normalised against the cost in order to determine the specific productivity of the process, which may be thought of as the price paid for a certain level of productivity. In this way the specific productivity may be thought of as combining both the previously discussed process economy metrics of productivity and also CoGs into a single parameter.

The usefulness of such a measure is that it can account for trade-offs between the productivity and process cost. For example multi-modal chromatography has a lower productivity than ATPE. It also however has a lower CoGs. Based on this, depending on which process economy metric is utilised, one technique may be thought of as being more attractive than the other. By normalising the productivity against the cost, and determining the specific productivity, it is possible to ascertain which is the most attractive, accounting for both parameters.

The specific productivity can be calculated either by dividing the productivity of the process by the cost, in which case the higher the value the better, or alternately the cost of the process may be divided by the productivity, in which case the lower the value the better. In this case the specific productivity was calculated by dividing the productivity of the process by the cost, with the initial absolute values expressed in dollars per gram per hour ($/g/h). These values were then normalised in the same manner as was done for both the productivity and CoGs values. These are summarised in Table 8.5

In the base case, Protein A packed bed chromatography has the lowest specific productivity. This might be expected since Protein A has the lowest productivity of all the unit operations in this process train, and also the highest CoGs associated with it. The remaining unit ops have specific productivities which are between approximately 10 - 20 times that of the Protein A step. The high specific productivity of the multi-modal and anion exchange chromatography steps is most likely a combination of them being operated in flow through mode, and also the fact that these resins are less expensive compared to Protein A chromatography. Interestingly, of the non-affinity

Table 8.5: Summary of the specific productivity of individual unit operations and overall process trains tested during experimental studies

	Process Train Specific Productivity						
Step	Train 1	Train 2	Train 2A	Train 1.1	Train 3	Train 4	Train 5
1	1.00 (PA)	8.49 (ATPE)	8.49 (ATPE)	1.00 (PA)	1.00 (PA)	4.20 (ATPE)	4.20 (ATPE)
2	-	1.90 (DF)	-	-	-	1.39 (DF)	1.39 (DF)
3	12.30 (MM)	18.20 (MM)	0.01 (CEX)	10.72 (MM)	0.39 (HPTFF)	0.19 (HPTFF)	14.45(MM)
4	9.99 (DF)	6.44 (DF)	3.04 (DF)	11.36 (DF)	-	-	14.86 (DF)
5	18.89 (AEX)	10.55 (AEX)	8.70 (AEX)	15.42 (AEX)	2.77 (MM)	1.56 (MM)	15.70 (AEX)
Overall	1.00	0.51	0.02	1.00	0.08	0.04	0.32

*P*A - Protein A, MM - Multi Modal, CEX - Cation Exchange, AEX - Anion Exchange, DF - Ultra/Diafiltration

chromatography steps, the ultrafiltration/ diafiltration step has the lowest specific productivity, hinting that perhaps of these three unit operations, this step is the most likely to become a bottleneck.

ATPE displays higher specific productivities than Protein A. This would indicate that ATPE would be better capable of handling increased productivity requirements. since increases in throughput may be gained at lower cost than compared to Protein A. The ATPE step in Process Train 5, which utilises the additional back extraction stage has a lower specific productivity that the ATPE step in Process Train 2. However the overall specific productivity of Process Train 5 is higher, likely due to higher yield achieved over the first ultrafiltration process, following the ATPE step. However the specific productivity of the base case platform process is still greater than that of any of the other process trains, incorporating the use of an alternative bioseparation technique. Process trains which utilise HPTFF all have very low specific productivities, mainly attributable to the low process yields and hence productivities of the HPTFF step.

The specific productivity parameter can be seen to be a useful measure as it accounts for both productivity and process costs in a single parameter, thereby allowing the attractiveness of a bioseparation technique to be expressed in terms of its overall

process economy.

8.4.5 Potential Performance

The process trains incorporating the use of HPTFF all had low associated process productivities and CoGs, due predominantly to the poor step yields achieved using this technique, as a result of the unexpectedly poor product retention displayed by the charged HPTFF membrane. It could therefore be said that the values for these process economy parameters are not entirely reflective of the true potential of a HPTFF process, operating correctly, since the product retention could have been increased by simply decreasing the membrane pore size. The question then is how would the specific productivities of Process Train 3 compare to that of the base case, if the HPTFF process had performed as expected.

In order to determine this, the process models for Process Train 3 were adjusted to represent a "correctly" functioning HPTFF process and an idealised version of the whole purification process. As a result, it was assumed that the process yield was actually 95%, rather than the 8% obtained during the experimental studies. The number of diafiltration volumes required was also reduced by 75%. The rationale for this is that the product pool following Protein A was found to have been relatively pure. Thus the level of impurity clearance required from the HPTFF process is relatively modest. As a result it would most likely be unnecessary to utilise the three step diafiltration procedure which was used. Indeed during the experimental studies, the HCP concentration was lower than 4 ppm following just the concentration stage of the process which is already below that which might typically expected in the final product. A further assumption was that no dilution of the Protein A product pool was necessary prior to loading onto the HPTFF process, since the neutralisation could have been performed using a relatively low conductivity base such as Tris, instead of NaOH.

Figure 8.1 shows a comparison of the specific productivities of the different process steps in both the base case process, and also Process Train 3. In both cases, the primary capture step was accomplished using Protein A chromatography. Intermedi-

ate purification was performed using multi-modal chromatography in the base case, whilst HPTFF was used in Process Train 3. Anion exchange chromatography was used for polishing in the base case, whilst multi-modal chromatography was used in Process Train 3.

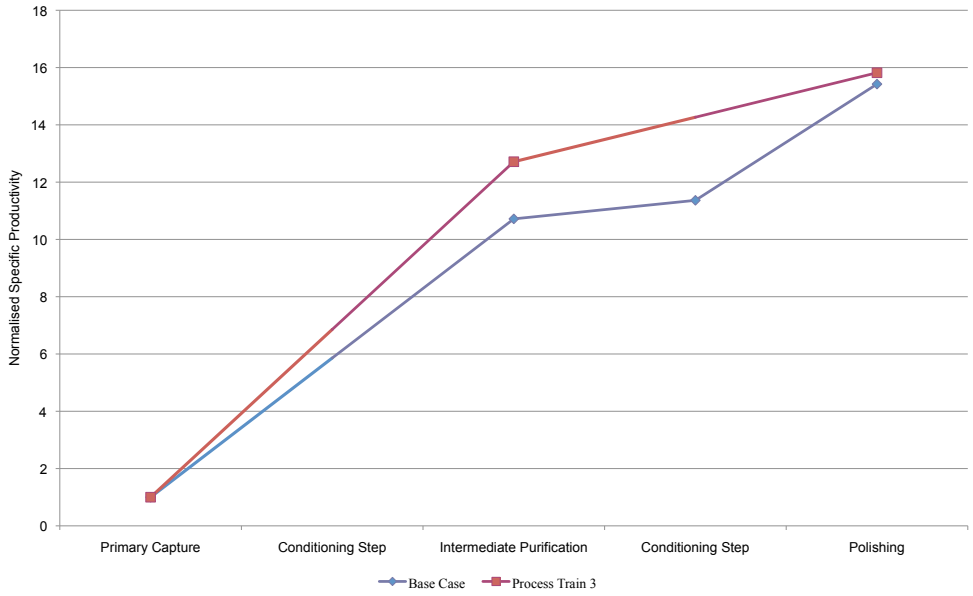


Figure 8.1: Comparison of specific productivity of steps in base case platform process and Process Train 3 under assumptions of ideal performance

The chart shows that HPTFF has a higher specific productivity than the multi-modal chromatography intermediate purification step in the base case. This is due to a combination of the shorter process time for the HPTFF step and also the relatively lower cost of the HPTFF membrane compared to the multi-modal resin.

The specific productivity of the polishing step in Process Train 3 is higher than that in the base case. This is mainly due to the higher product concentration of the load in Process Train 3, since the HPTFF step was also used to concentrate the product. Process Train 3 also does not make use of a conditioning step between the intermediate purification and polishing steps.

As a result of a combination of all of these factors, the overall specific productivity of Process Train 3 is approximately 30% greater than that of the base case

process. The use of a correctly functioning HPTFF step can therefore be seen to have some benefits in terms of process economy. It could be argued that the presence of the conditioning step is not completely necessary since the pH following multi-modal chromatography could simply be increased using a low conductivity titrant. Removal of this conditioning step reduces the specific productivity advantage of Process Train 3 over the base case from 30% to 10%. These results would therefore indicate that incorporation of a "correctly" functioning HPTFF step into a platform process can result in some slight improvements in the economics of the process. The performance of Process Train 3 in terms of HCP clearance would indicate that a product of sufficient purity can be obtained. The only issue is the poor step yield which was displayed by the HPTFF process. This could in theory be simply corrected by altering the membrane MWCO. This may however affect the flux achievable through the membrane, which in turn would negatively impact the specific productivity. Further experimental studies using a membrane with a smaller pore size would therefore need to be performed in order to determine whether the performance of the HPTFF containing process train can still match that which has been modelled here.

This example of an "ideal" Process Train 3, shows the potential benefits which may be gained from using a correctly functioning HPTFF process. The same hypothetical analysis could be applied to Process Train 5. This process train, whilst not exceeding base case process, did show the most comparable specific productivity. In this case, the ATPE process was hindered by the need for additional back extraction steps and also the relatively low process yields achieved across the diafiltration process directly following ATPE. As was discussed previously, the utilisation of multiple back extraction steps was not included in the original ATPE system described by Andrews et al.[110] The process models were therefore adjusted, with the assumption that product could successfully be recovered in the bottom phase following a single back extraction step. The yield from the diafiltration step following ATPE was also increased to 95%. It is not feasible to remove the diafiltration step completely due to the very high conductivity of the bottom phase form the ATPE step. It would be unfeasible to load material with that high an ionic strength onto the following multi-

modal chromatography step, as this would most likely prevent any impurity binding. Figure 8.1 shows a comparison of the specific productivities of the different process steps in both the base case process, and Process Train 5.

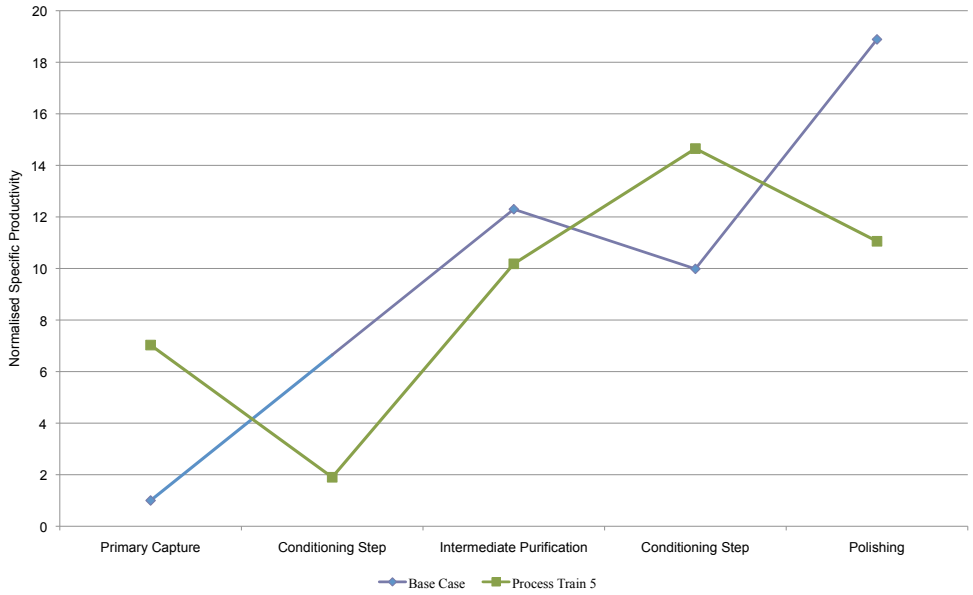


Figure 8.2: Comparison of specific productivity of steps in base case platform process and Process Train 5 under assumptions of ideal performance

From this it can be seen that whilst the specific productivity of the ATPE step used for primary capture in Process Train 5 is higher than that of Protein A in the base case, the specific productivity of the remaining steps are actually lower, with the exception of the second diafiltration step between the multi-modal and anion exchange chromatography steps. The specific productivity of this step is higher in Process Train 5 due to the higher concentration of the feed material, leading to a higher productivity (the first diafiltration process following ATPE was modelled to have concentrated the product). The specific productivities of both the intermediate purification and polishing steps are due to the lower capacities for these two processes in the ATPE incorporated process train. This was done in order to account for the higher impurity profile of the ATPE processed material. The diafiltration step following ATPE for product stream conditioning, is not present in the base case, and it would seem

that the addition of this step has had a deleterious effect upon the overall specific productivity of Process Train 5, which even under these "idealised" conditions is still only approximately 70% of that of the base case process. Removal of this diafiltration step, with the assumption that the bottom phase from the ATPE process could be directly loaded onto the multi-modal chromatography column, actually increases the specific productivity of Process Train 5 to being over 80% greater than that of the base case. The switch from 30% below the specific productivity of the base case, to 80% greater, through removing the diafiltration step following the ATPE process shows just how much of a bottleneck this step is. The feasibility of doing this however is questionable given the conductivity of the bottom phase from the ATPE process. Even taking into account the fact that multi-modal resins have been designed to be capable of operation at relatively high ionic strength conditions, the salt concentrations are such that at least a 3 fold dilution of this phase would be required in order to reach manageable levels of conductivity.

Figure 8.3 summarise the overall specific productivity of Process Trains 3 and 5 under these new sets of assumptions.

8.4.6 Process Economy in Light of Increasing Product Titre

A major potential advantage inferred by ATPE is the fact that it may not present the same capacity and productivity constraints as Protein A chromatography. In order to examine this, additional processing scenarios were analysed in which the cell culture product titre was increased from 5g/L up to 10g/L, 15g/L and 20g/L, with the impact of these increases in concentration upon the specific productivities of the base case process and Process Train 5 observed. The chromatography steps were re-sized as appropriate to handle the increased amount of product in the feed, whilst it was assumed that no adjustments would have to be made to the ATPE system volume ratios.

Process Train 5 was modelled under a number of different configurations. In the first configuration, the process was modelled with the use of the ultrafiltration/diafiltration step immediately following ATPE, in order to effect buffer exchange

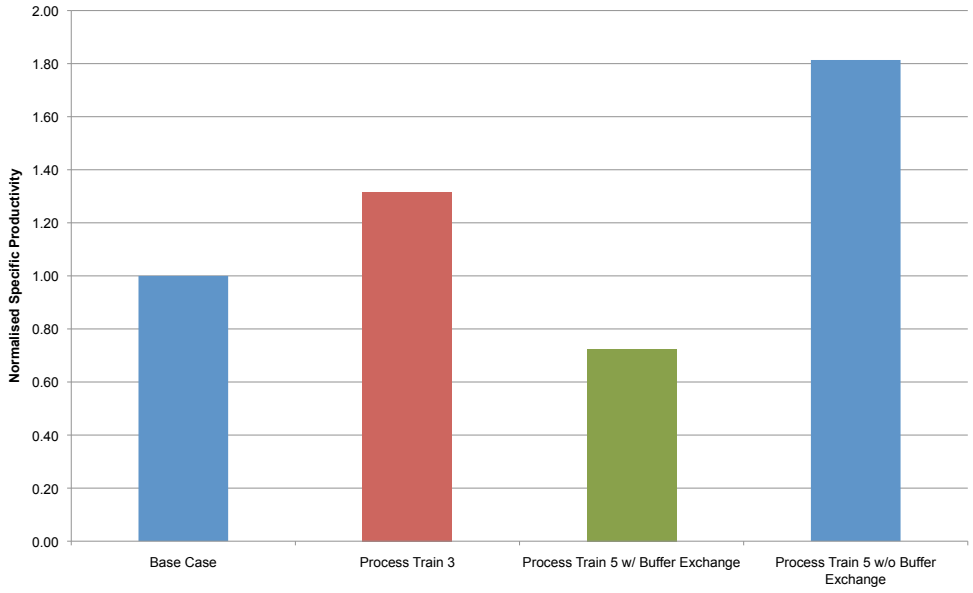


Figure 8.3: Comparison of overall specific productivity of Process Trains 3 and 5 compared to the base case process

prior to the intermediate purification step. In the second configuration, this buffer exchange step was removed, and it was assumed that the bottom phase from the ATPE process could be directly loaded onto the multi-modal chromatography column. In the final configuration, the ultrafiltration step following ATPE was also removed, however it was assumed that a 1 in 3 dilution would need to be performed in order to reduce the conductivity to a sufficient extent that loading onto the intermediate purification step could be permitted. The buffer exchange step following the multi-modal step was then used to achieve a 3-fold concentration factor to compensate for this dilution.

Figure 8.4 shows a plot of the specific productivity of these Process Train 5 configurations, normalised against that of the base case platform process, as a function of the product titre in the feed.

At a feed concentration of 5g/L, it can be seen that only the configuration in which the ultrafiltration step following ATPE is removed, is the specific productivity of Process Train 5 greater than that of the base case. However at product titres of approximately greater than 6 g/L, the specific productivity in all configurations is

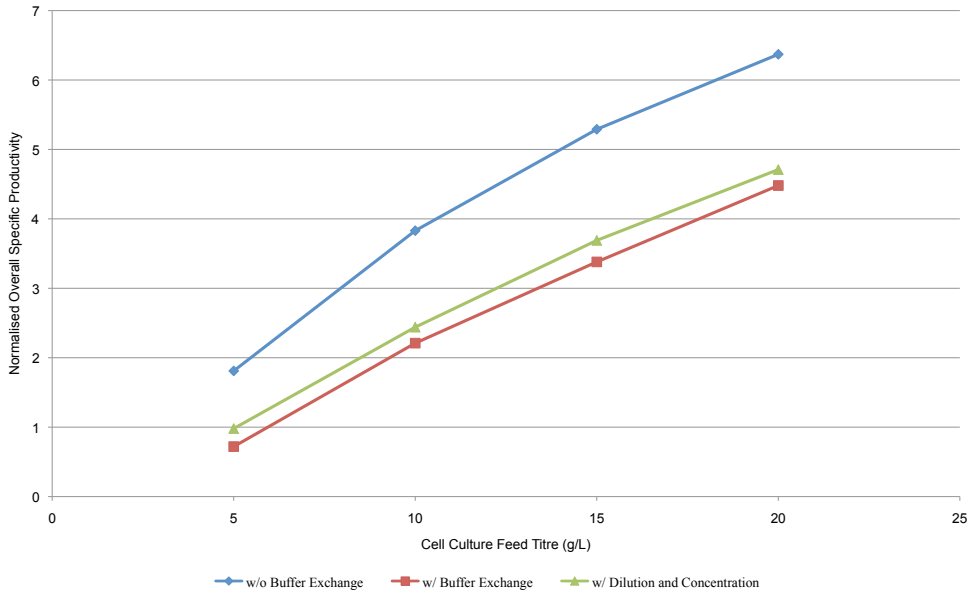


Figure 8.4: Comparison of overall specific productivity of different Process Train 5 configurations as a function of the cell culture feed titre

greater than that of the base case. Indeed by the time the feed concentration has reached 10g/L, the specific productivities are over 2 times that of the base case.

The aim of this study was to quantify the benefits which may be conferred through the utilisation of ATPE and HPTFF. The results presented in Figure 8.4 would suggest that these benefits only become evident once the titre has increased beyond 5g/L at which point potential capability of ATPE to scale with the process volume rather than the mass of product which needs to be purified, helps to provide the observed increases in process productivity.

Such a result comes with the significant caveat that product solubility limits are not reached during the ATPE process at such feed concentrations. This is a fairly significant assumption, and based on the product precipitation issues which were encountered during the development of the ATPE process, it is unlikely that these limits would not be reached. Thus whilst it would seem that at a feed concentration of 15g/L, a process train based around the use of ATPE might be able to provide a specific productivity which is over three times greater than that of a Protein A based

platform process, there may be thermodynamic limits which reduce the feasibility of operating an ATPE step at this concentration.

If it is assumed however that operation up to concentrations of 20g/L are feasible, the results in Figure 8.4 presents the process engineer with conditions under which the utilisation of ATPE could potentially be advantageous. For example if the product titre in the upstream cell culture process is 5g/L or less, the use of ATPE in place of Protein A chromatography does not yield any productivity benefits. As a result if a biomanufacturer is commonly achieving cell culture titres around this level, then the benefits of adopting ATPE are most likely not sufficiently high to justify its adoption. This is because, even though the productivities are comparable, the utilisation of ATPE has implications upon other aspects of a mAb development and manufacturing cycles. For example, as was described in Chapters 6 and 7, the nature of ATPE means that new systems will need to established from scratch with each new product entering process development. In the case of the ATPE system which was utilised in these studies, the development of the process, to the point where it could actually be applied to the purification of an antibody product took over three months. Development, or more correctly optimisation of a Protein A process, due to its robustness, could likely be completed within 2-3 weeks. Thus for 3 new antibody products, Protein A processes could be developed within a cumulative period of just over 2 months, whilst development of ATPE processes for each product, could collectively take up to 9 months. High throughput screening approaches to process development could help to reduce these process development timelines for ATPE, however even then times could still be long, as the bottleneck is simply shifted to analytical departments. In light of this, given the choice between a Protein A based process and an ATPE based process, biomanfacturers would most likely go for the former if both processes exhibit comparable productivities. At titres of greater than 5g/L, the use of ATPE begins to confer increases in the the specific productivity over the base case process due to a decrease in the CoGs and also an increase in the relative productivity compared to Protein A chromatography.

The question then becomes, at which point do the potential productivity benefits

outweigh the increased timelines associated with process development. This is a difficult trade-off to weigh, since process development and manufacturing occur at different points of a drug's lifecycle and as a result direct comparisons in terms of time and cost are difficult to make. Ultimately the decision of whether to adopt ATPE will depend upon the specific processing circumstances of the biomanufacturer. This will include the number of products which need to be manufactured by the facility and the associated level of demand. The data shown in Figure 8.4 however does provide guidance as to when the replacement of Protein A with ATPE could be considered, particularly in light of processing constraints. For example at a cell culture titre of 10g/L, the total process time for the Protein A base process is approximately 40 hours. If the plant scheduling is such that a certain number of process batches must be performed in order to meet product demands, and this in turn imposes a 24 hour constraint upon the downstream processing time then it may be feasible to utilise an ATPE process, which at this product concentration has a productivity, double that of the base case (and therefore a process time of approximately 20 hours). As stated it is highly dependent upon the constraints of the biomanufacturer, since for example the productivity of the Protein A based process could be increased by simply increasing the number of Protein A chromatography columns and running them in parallel. However this would increase the plant space requirements.

Another consideration is plant flexibility. In terms of productivity, the ATPE process can be seen to be capable of handling a greater range of cell culture titres, particularly if there are constraints placed upon the minimum level of throughput required. On the other hand Protein A has an advantage in terms of technical flexibility, since as was described in previous chapters, the variable nature of the performance of ATPE will mean that it cannot be guaranteed that it will work for every single product. Therefore the decision as to whether to move towards the replacement of Protein A with ATPE would need to factor in which type of flexibility has a greater level of value. A plant capable of handling large variations in feed titre or one which is almost guaranteed to be capable of producing a large range of different antibody products.

8.5 Conclusions

The results of the experimental studies detailed in Chapter 7 showed that the performance, in terms of yields and impurity clearance, of process trains incorporating the use of alternative bioseparation techniques, were unable to match that of the base case Protein A platform process. Based on this it could be argued that these technologies are not genuine process alternatives, at least from the perspective of mAb purification. Such an argument however does not account for the potential process economy advantages which may be conferred through the used of these alternative techniques. Indeed a major selling point for many of the alternative techniques currently being considered is the potential for providing higher process productivities at reduced costs.

The study detailed in this chapter was undertaken in order to quantify the benefits, in terms of the process economy, of adopting these technologies. Techniques have been compared in terms of a number of process economy metrics shows that whilst some advantages in terms of productivity of CoGs may be attained through the use of these alternatives, consideration from a whole process perspective would indicate that based on the performance of these techniques observed during the experimental studies detailed in Chapter 6 the overall process economy advantages are not significant, if indeed at all existent.

In the case of ATPE, any process economy advantages conferred by the technique itself, were severely compromised by the need for product stream conditioning following ATPE in order to integrate this step into the process. These findings clearly illustrate the danger of evaluating the attractiveness of a bioseparation technique in isolation, rather than as part of a whole process train. The ATPE process alone seems like a fairly feasible alternative to Protein A chromatography. It has comparable CoGs, but also has a much higher level of productivity. The overall benefit in process economy is however negated by the need for the diafiltration step prior to the subsequent unit operation in the downstream process.

HPTFF was seen to provide slight process economy benefits over the base case.

However this was based on assuming that it would behave in the manner in which it was expected, rather than in which it was actually observed. Further work would need to be performed in order to accurately determine whether the advantages in terms of productivity and cost, provided by this technique can truly be delivered. It would therefore seem that whilst the performance in terms of recovery and impurity clearance of these alternative process trains was not able to match that of the base case, the process economy advantages provided are also not of a sufficient magnitude.

Modelling of idealised cases, in which the performance of these unit operations was "boosted" to reflect their potential performance, resulted in only relatively small improvements in specific productivity. Sizeable benefits in terms of process economy were however observed upon increasing the cell culture titre. In the process train in which ATPE was used to replace Protein A chromatography, the specific productivity was observed to increase proportionally to the product titre. Based on the results from this part of the study, it was observed that productivity benefits would only be conferred through the use of ATPE at cell culture titres of greater than 5 g/L. This result however must be caveated with the point that the performance of the ATPE process was modelled as what might be achieved, rather than what was exactly observed during the experimental studies.

An argument previously put forward was that whilst the yields and impurity clearance achieved using these techniques were not able to match that of the base case platform process, they were also not sufficiently low in certain cases, so as to suggest that the performance could not be brought up to the level of the base case through increased process development effort. The issue however is that because of this, the adoption of a technique such as ATPE would essentially rule out the possibility of utilising a platform approach to mAb purification. The lack of robustness displayed by ATPE during the experimental studies would suggest that certain situations would be encountered in which the use of this technique would become unfeasible, in which case an alternative primary capture process would need to be used. As a result, locking down ATPE for primary capture, which would be necessary for establishing a process platform, is not possible. The question then is whether abandonment of

a completely platform approach to downstream processing could be justified by the economic advantages provided by ATPE.

Extrapolation of the experimental results to predict what might be possible shows that some benefits may be gained. The size of these benefits and their significance however are highly dependent upon the circumstances of the biomanfacturer. For example the model result show that at product titres of 10g/L, the process train in which Protein A is replaced with ATPE is capable of achieving a specific productivity, double that of the base case. This is only a significant result if titres of 10g/L are being commonly achieved by upstream processes. If in fact such titres are uncommon, then the benefits achieved using ATPE will rarely be encountered. It will also depend upon whether the doubling of productivity is significant in terms of process scheduling constraints. The high relative productivity display may imply that ATPE may find applications in niche processing scenarios in which high process throughputs are required from a facility dedicated to the manufacture of a single product. Importantly, it must be considered that the results showing increased relative productivities were based on assuming that the ATPE process could operate at such high product concentrations, and that solubility limits are not reached. This is a particularly significant assumption when taking into account the product precipitation issues encountered when developing this technique was was detailed in Chapter 6. As a result the observed improvements must be treated as being somewhat hypothetical.

Ultimately the only clear conclusion which may be drawn, based predominantly upon results obtained using data collected from the experimental studies detailed in Chapter 7, is that the abandonment of the platform approach cannot be justified, since the economic advantages conferred by the use of alternative bioseparation techniques over the Protein A based platform process were not of a significant magnitude.

Chapter 9

Conclusions

The general aim of this thesis was to evaluate the potential challenges posed by alternative bioseparation techniques to the ensconced nature of packed bed chromatography in the biomanufacturing of biomolecules. In particular, the aim was to evaluate the feasibility of adopting these techniques into the purification of mAb products, the advantages which they may confer and also the potential drawbacks.

The conclusion which may be drawn from the results of the studies which have been performed is that whilst alternative may indeed offer potential advantages in terms of productivity and cost, these advantages are not sufficient to outweigh the drawbacks associated with their adoption.

9.1 Constraints of Platform Processes and the Need for Alternative Technologies

As was discussed in Chapter 2, packed bed chromatography and more specifically Protein A chromatography has become a consistent feature in almost all mAb purification processes. In light of advances in upstream operations concerns were raised regarding the potential for packed bed chromatography to become a constraint on both process productivity and cost. In response to this, one of the ways in which it has been suggested that such constraints may be overcome is through the use of alternative bioseparation techniques, offering higher throughputs and lower cost. Chapter

3 gave an outline of just some of the bioseparation techniques currently being considered. It could be seen from this outline that these alternatives consist of a wide range of techniques, utilising a number of different mechanisms of separation. They however all have the common characteristic of potentially being able to overcome the cost and capacity issues presented by packed bed chromatography.

The potential of these alternative techniques is clearly not lost on biopharmaceutical manufacturers. Results of a survey circulated to individuals involved in the development and manufacture of bioproducts, detailed in Chapter 4 revealed that there is a significant level of interest in their use. The survey results also however suggested that this interest was tempered by a certain degree of caution. Increasing competition in the biopharmaceuticals market has meant that organisations are continually reducing development timelines in order to increase the speed to market of their products. This in turn means that there is less time available for process engineers to investigate the use of novel technologies. As a result cases in which alternatives had been incorporated into alternative bioseparation techniques were mainly limited to the use of techniques which were variations on established technology, such as for instance membrane chromatography, which essentially operates in the same manner as packed bed chromatography, albeit with the use of an alternative form of stationary phase support.

9.2 Theoretical Evaluation of Alternative Technologies

It could be argued that such caution prevents biopharmaceutical manufacturers from investigating alternatives which have the potential to truly alter current biomanufacturing paradigms in such a way as to eliminate the possibility of downstream processing ever becoming a bottleneck, even in light of the advances in upstream technologies. To determine whether this was the case, a MADM based framework was developed, designed to evaluate the "industrial attractiveness" of a given bioseparation technique, accounting for the trade-off between different qualitative and quantitative process at-

tributes. This approach was designed to assess the alternatives in completely rational terms, free of subjectivity and unclouded by notions of perceived risk. The conclusion drawn from this study, detailed in Chapter 5 was that none of the alternatives are currently able to match the attractiveness of packed bed chromatography. Furthermore, manipulation of the non-deterministic outputs from the framework showed that the performance of even the most industrially attractive alternative techniques, must be pushed significantly beyond that which has to date been observed, in order for them to match the levels of conventional chromatography.

The use of the MADM based framework did however enable ATPE and HPTFF to be identified as the most attractive alternative bioseparation techniques. And the aforementioned manipulations of the non-deterministic outputs of the model did indeed discern the key areas in which process development on these techniques should be focused, in order to improve their relative attractiveness compared to Protein A chromatography to be determined as well as providing tangible targets for this development. However as stated, these targets were for the most part beyond that which may currently be accomplished.

A significant caveat of this analysis is that the industrial attractiveness of these techniques was determined based on their use in isolation, rather than as part of a whole process train. This may have resulted in a underestimation of the potential of these techniques. For instance, the majority of alternative bioseparation techniques were unable to match the purification performance provided by Protein A chromatography. Purification factor was identified by survey respondents as being a process attribute of an extremely high level of importance. This is not surprising given the purity requirements of all pharmaceutical products. However in such cases, it is the purification performance of the downstream processing train as a whole rather than that of the individual unit operations of which it is comprised, which is of the greatest importance. Thus even if an alternative with a relatively low capability for impurity clearance were used, as long as the remaining unit operations were capable of providing a sufficient level of purification, then the low level displayed by the alternative would be almost irrelevant.

9.3 Practical Evaluation of Alternative Technologies

Experimental studies were therefore performed in order to examine the performance of the two most industrially attractive alternative bioseparation techniques, when used as part of a whole process train. The results of this study, detailed in Chapters 6 and 7 showed that the yield and impurity clearance achieved using process sequences which incorporated the use of an alternative bioseparation technique, were not able to match that of a Protein A based platform process. The study also revealed another danger of considering techniques in isolation, which is that in such cases, little to no account is made of how these alternatives may potentially interact with other unit operations. A prime example of this was the need for a protracted buffer exchange operation following ATPE in order to condition the feed stream to make it suitable for subsequent steps in the process.

Process robustness was a key observation made during the experimental studies. Robustness in this case, rather than referring to the ability of a technique to handle slight variations in feed conditions, is used in a more general sense, alluding to how the process is able to cope with a variety of different products of the same modality. In this sense, Protein A is highly robust for the purification of mAb molecules, since it is capable of capturing practically all IgGs from almost any given feed stream. ATPE and HPTFF did not show the same level of robustness, with the performance of these two techniques deviating significantly as a result of changes in the feed material. Such a finding highlights a key issue with alternative bioseparation techniques. The initial concept behind the adoption of alternatives was that they could potentially facilitate a paradigm shift in biomanufacturing, such that downstream processing was no longer a constraint on the productivity of a facility. The observed lack of process robustness, displayed by ATPE and HPTFF, would suggest that such a shift, if one were indeed to occur, would be away from a platform approach to mAb purification. The only matter then is whether such a move could be justified.

9.4 The Future Potential of Alternative Technologies

Whilst the yield and impurity clearance performance of these alternative processes were not sufficient to match that of the base case Protein A based platform, it could be argued that such deficiencies could be overcome through increased process development effort. Indeed, even though the performance of the process trains incorporating the use of ATPE and HPTFF did indeed fall short of that of the base case platform process, the degree by which they fell short was not a sufficient magnitude, that their feasibility could be rule out. Instead the question of whether or not to adopt alternative bioseparation techniques, comes down to the process economics. As was discussed in the initial review of these technologies, ATPE and HPTFF have the potential to offer higher process productivities and at lower cost compared to packed bed chromatography. The only issue then is whether the benefits in terms of these process economy related metrics are sufficient to justify movement away from a platform based approach to mAb purification. For example it may be possible to utilise ATPE to purify 4 out of 5 different antibody products, with the fifth product requiring a change in the manufacturing process and a move back to a Protein A based platform. The question is whether the time and cost saved in processing the first four products, outweighs the time and cost investment required in order to move back to Protein A for the final, fifth product. The analysis of process economy detailed in Chapter 8 would suggest that this is not the case.

The results of this analysis revealed that whilst incorporation of both ATPE and HPTFF did confer increases in productivity and associated CoGs over the base case Protein A platform process, these increases were not of an overwhelming magnitude. Indeed, even under idealised model conditions in which the performance of the alternative techniques were extrapolated to that which might be expected following sufficient process development, only incremental increases in productivity and CoGs were observed. This hardly puts forward a compelling case for the adoption of alternative bioseparation techniques.

9.5 Advantages of the Platform Approach

As alluded to previously, a major advantage of mAb purification platform processes, built around the use of packed bed chromatography, and in particularly Protein A, is its adaptability and robustness. This facilitates more rapid process development, since the unit operations which need to be optimised are essentially "locked-in" place. It also makes the design of manufacturing facilities much more straightforward, particularly in the case of those which need to be able to process multiple products. Based on variability in the performance of ATPE and HPTFF, it would seem that the adoption of such alternative technologies would require a movement away from a platform approach to mAb purification. The hope is that the benefits provided by the utilisation of these techniques would outweigh the deleterious impact of abandoning platform processes. Whilst preliminary qualitative evaluations predicted that these techniques could be used to provide significant increases in productivity and reduced costs, the results of all the quantitative analyses which have been performed, show that the level of benefit conferred through the use of alternative techniques such as ATPE, is only apparent once a certain threshold is passed in terms of the product titre of the cell culture process. Based purely upon the results achieved over the course of this study, it would seem that the productivity benefits are not sufficiently large as to make an overwhelming case for the widespread adoption of ATPE in place of Protein A, particularly in light of the difficulties encountered during process development.

9.6 Sustainability of Current MAb Manufacturing Paradigms

This thesis aimed to evaluate the potential challenges to the ubiquitous nature of packed bed chromatography, and in particular the challenge posed by so-called alternative bioseparation techniques, in light of the advances seen in upstream operations. The results which have been obtained over the course of this study, suggest that these challenges are fairly modest. The widespread adoption of alternative techniques such

as ATPE and HPTFF is hindered by the lack of adaptability to different feed materials. The potential for significant increases in productivity will most likely only be attained at increased product titres and even then this is based on the extrapolation of the performance of these techniques to what may be achievable, rather than that which was actually observed. The decision by a biomanufacturer, of whether to pursue the adoption of an alternative bioseparration technique, over the use of conventional packed bed chromatography is therefore dependent upon what process attribute has a greater value. On the one hand ATPE for instance potentially provides a greater degree of flexibility in terms of being able to handle the increases in product titre predicted from upstream operations, at the expense of possibly not being applicable over a wide range of products. The Protein A based platform process on the other hand may place constraints upon the maximum productivity which may be achieved, but comes with the advantage of high levels of adaptability and robustness.

Tellingly, the majority of respondents to the survey detailed in Chapter 4 felt that the future challenge for downstream processing was not in the development of processes capable of generating multi-ton quantities of product, but instead lay in the establishment of facilities capable of manufacturing multiple products. Such a challenge, emphasises the need for robust process platforms rather than for inherently variable processes capable of high process productivities. This is mainly due to the fact that the age of blockbuster biopharmaceuticals is potentially coming to an end. The relatively large number of biopharmaceutical products which have reached blockbuster status would indicate that perhaps all the low hanging fruit has been picked. Instead biomanufacturers are looking towards the development of a larger drug development pipelines and product portfolios of greater breadth, aiming these therapeutics at an ever dwindling population of indications. In light of this, it is no surprise that competition in the field of biopharmaceuticals is increasing, making speed to market a progressively important consideration. As a result biomanufacturers are forced to condense process development timelines, further emphasising the need for robust and adaptable process platforms. In the case of mAb manufacturing these driving forces all lead towards a continued reliance on packed bed chromatography.

Chapter 10

Future Work

The overall results from this study indicate that the widespread use of alternative bioseparation techniques is currently not feasible due to the current technical limitations of these technologies. However based on the results which have been obtained over the course of this study, there are a number of areas in which further work could be performed in order to better understand these limitations and the ways in which they may be overcome.

10.1 Process Development

For example, the results of the non-deterministic analysis performed in Chapter 5, highlighted the key areas in which many of these alternative bioseparation techniques are falling short of packed bed chromatography. The head to head comparisons between ATPE, HPTFF and Protein A chromatography showed the processing attributes in which process development should be focused in order to improve the industrial attractiveness of these alternative techniques so as to make them genuinely viable processing options. One potential avenue for future work would therefore be to try to develop ATPE and HPTFF processes with improved performance in the areas identified from the MADM analysis in Chapter 5.

The ATPE and HPTFF processes evaluated in Chapters 6 and 7 were based on those detailed in the literature. These processes underwent only a limited level of

process development and as a result, it could be argued that the problems encountered in terms of the performance of these techniques were inherent to the process parameters which were used. For example the performance of the ATPE and HPTFF process could have been dramatically improved by switching to a higher molecular weight polymer and a membrane with a smaller molecular weight cut-off respectively.

Further work could therefore potentially involve the development of an ATPE process from scratch, utilising a design of experiments approach for both screening and optimisation of conditions. Due to the limited level of understanding with regards to the partitioning behaviour of biomolecules in ATPE systems, development of high throughput screening methods would be required, possibly in 96-well plate format. Experiments at such scale would pose its own challenges, particularly when it comes to phase separation and recovery. However a clean sheet approach such as this would potentially enable a more efficient ATPE process to be developed, with an industrial attractiveness comparable to that of affinity packed bed chromatography for mAb capture.

10.2 Process Robustness

Process robustness was a key finding from the experimental studies detailed in Chapters 6 and 7. Both the ATPE and HPTFF systems evaluated, displayed a certain lack of process robustness, with their performance deviating with the use of different feed materials. This is a key drawback to using these alternative techniques, as it make the utilisation of a platform approach to downstream purification, difficult. A possible area worthy of further investigation, is to determine whether this lack of robustness is indeed common to all systems or whether the problems encountered were simply due to a particularly difficult IgG product, displaying atypical behaviour for a molecule of this modality. This could be done by repeating the studies described in Chapters 6 and 7 using, rather than different batches of the same product, a range of different IgG products.

It could of course be argued that even if the IgG used for the experimental studies

was atypical, since the standard Protein A base case platform process still worked, then the poor performance ATPE and HPTFF shows that these techniques do not display a sufficient level of robustness to be used in a platform process. However it would still be of interest to disseminate the actual level of robustness associated with these alternatives. For example the ATPE system evaluated in this study may perform perfectly well for 9 our of 10 IgG products, with the IgG used in this study being the exception. Alternatively, the ATPE system may only be compatible with 1 in 5 antibody products, in which case the IgG used in this study would actually fall into the majority. Further experimental studies would also need to include the use of multiple different ATPE systems.

10.3 Increased Product Titre

Another area which could be investigated, is how these techniques behave at increased product titres. An interesting result which was obtained during the process economy modelling of these techniques, was the potential ability of ATPE to handle increases in product titre without compromising productivity. Such an ability means that ATPE could potentially find a use in certain processing circumstances in which high process throughputs are required. As was stated however, this potential is based upon a number of assumption, a key one being the fact that product solubilities limits are not reached as a result of the increases in feed concentration. The experimental studies which were performed, utilised feed material with a relatively low product titre of between 1 to 5g/L. Further work could involve repeating the experiments involving the use of ATPE, with feed material containing IgG at concentrations of 10g/L and higher. This would then provide insight as to whether ATPE processes can actually be operated at such IgG feed concentrations, without encountering issues with product precipitation.

10.4 Cost of Development

The results of the economic modelling performed in Chapter 8 showed that whilst benefits in terms of productivity and CoGs may be achieved during manufacturing with the use of ATPE and to a lesser extent HPTFF, these savings do not reflect the level of additional process development effort which may potentially be required. For example ATPE may be capable of providing a 1$/g saving in the CoGs when compared to Protein A chromatography. However the implementation of ATPE step into a manufacturing process may require an additional 3 months of development work. The question then becomes whether the additional development time, and of course the associated development costs, can be justified by the reduction in the CoGs. This is a difficult comparison to make since the scales at which costs are measured during development are significantly different to those used during commercial manufacturing. Such a comparison would need to account for a number of factors related to the commercial value of the IgG product, such as the market size, projected market share, annual demand and campaign scheduling. Business related strategic factors would also need to be accounted for, such as the existence of competitors and their relative progress in clinical development.

The development of a methodology or framework, which would enable such comparisons to be made, could form the basis for further work. Such work would require in depth economical analysis and projection of revenues balanced against development and manufacturing costs. Scenarios could then be modelled. It could be envisioned that such modelling would enable a "tipping point" to be established, beyond which additional development costs cannot justify the associated reduction in the CoGs or productivity. Establishing such a point would enable a deeper understanding as to the feasibility of adopting an alternative bioseparation technique.

10.5 Alternative Bioproducts

This work has primarily focused on the use of ATPE and HPTFF and alternatives in general in the field of MAb manufacturing. This scenario was chosen as the mAb

market is currently the major sector of the biopharmaceuticals markets. Future work could involve the investigation of the use of these techniques in the manufacture of growing sectors such as vaccines, and how these compare against more conventional unit operations. Such products are not as mature as MAb manufacturing and as such do not have associated platforms. It may therefore be of interest to determine whether the performance of alternative techniques are sufficiently robust to enable platform processes for such products to be developed.

Chapter 11

Appendix A: GE Healthcare Letter

In Chapter 5, a study was alluded to, in which the non-deterministic outputs of the MADM based framework, was used to compare non-specific and Protein A affinity chromatography. The purpose of this was to demonstrate a possible business oriented application of the MADM tool. Overleaf is a letter from GE Healthcare, presented in lieu of the precise details and results of this study.

Chapter 12

Appendix B: Patent Application

Aqueous Two Phase Extraction Augmented Precipitation Process for Purification of Therapeutic Proteins

Inventor(s): Richard Tran, Nigel J. Titchener-Hooker, Karol M. Lacki

12.1 Introduction

In Chapter 6, details were given regarding the unexpected behaviour of the ATPE system during the back extraction step, using the conditions developed by Andrew et al. [110]. During this step, mAb was observed to precipitate at the interface between the two phases of the ATPE back extraction system. Preliminary characterisation of this interfacial precipitate revealed that it had a high IgG content, and a relatively low impurity composition. As a result, the back extraction system was indeed causing a purification of the product, albeit in a different manner to that which was expected. The ATPE step was therefore behaving as a hybrid of two separate alternative separation techniques; ATPE and protein precipitation. The ATPE augmented precipitation process had an advantage over standard protein precipitation, in that the ATPE forward extraction provides a single pass of purification as some impurities presumably partition into the bottom phase of the ATPE system. The augmented precipitation process also has an advantage over standard ATPE processes in that the product is recovered in the form of precipitate, and can therefore theoretically be resolubilised in any desired buffer system, making integration of the ATPE process more straightforward than was observed in the studies detailed in Chapter 7. Furthermore the concentration factors which may be

achieved as a result are also higher than those which might typically be expected from an ATPE process. In light of the potential for applying this ATPE augmented precipitation process, for large scale biomanufacturing, a patent application was filed. This patent application is detailed in this chapter.

12.2 Abstract

The invention relates to an aqueous two phase extraction (ATPE) augmented precipitation process, which may be used to recover and also partially purify therapeutic proteins, including monoclonal antibodies from a crude multi-component mixture. The process involves the formation of a forward extraction PEG-Phosphate ATPE system in which the target product is preferentially partitioned to the polymer rich phase. A second ATPE back extraction system is then formed by introducing the polymer rich phase from the forward extraction to a new phosphate salt rich phase, causing the product to precipitate at the interface between the two phases. This precipitate is then recovered and resolubilised in a suitable buffer and may be passed on for further purification.

12.3 Field of the Invention

The present invention relates to the field of protein purification and in particular, methods for capturing and purifying proteins from crude multi-component mixtures. Specifically, it relates to the use of a combination of an aqueous two phase extraction system and a protein precipitation process, in order to affect bioseparation of the target molecule.

12.4 Background of Invention

Monoclonal antibodies (mAbs) currently represent the most prevalent biopharmaceutical product in either manufacture or development by organisations worldwide (see Jacobi A, Eckermann C and Ambrosius, Bioseparation and Bioprocessing 2nd Edition Volume 2 2007 Wiley-VCH p 431). The high commercial demand for and hence value of this particular therapeutic market has lead to the emphasis being placed on pharmaceutical companies to maximise the productivity of their respective mAb manufacturing processes whilst controlling the associated costs. Technological developments upstream have gone some way towards addressing this challenge with advances in mammalian cell culture technology resulting in typical mAb titres rising from tenths of a gram per liter, up to 10 grams per liter, over the past decade (see Thommes J, Etzel M. Alternatives to Chromatographic Separations, Biotechnology Progress 2007; 23: 42-45). Along with these higher product concentrations, advances in bioreactor technology have meant that companies now operate cell culture

processes with volumes of up to 25,000L (see Kelley B. Very Large Scale Monoclonal Antibody Purification: The Case for Conventional Unit Operation, Biotechnology Progress 2008; 23: 995-1008). Whilst upstream operations have advanced in order to meet the challenges of increasing product demand, it has been argued that the developments in downstream processing (DSP) technology have not kept pace. The downstream purification of the majority of mAb products, either marketed or in development, is currently based on the utilisation of process platform built around the use of Protein A affinity chromatography (see Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. Downstream processing of monoclonal antibodies - Application of platform approaches, Journal of Chromatography B 848: 28-39 and see Sommerfeld S and Strube J. Challenges in biotechnology production - generic processes and process optimisation for monoclonal antibodies, Chemical Engineering and Processing 2005; 44: 1123-1137). This heavy dependency upon the use of Protein A for mAb purification has been the cause for some concern amongst process engineers involved in the purification of mAb products. Protein A chromatography processes are scaled based upon the mass of product which needs to be captured. A 10,000L fermenter may be used to culture a cell line expressing mAb at a concentration of 1g/L, thereby producing a total of 10kg of mAb per batch. If the same fermenter is used to culture a cell line expressing mAb at a concentration of 10g/L, the volume of the cell culture will still be 10,000L, but the mass of mAb contained within will now be 100kg. Thus the Protein A chromatography column will need to be either made 10 times as large as that which was used to capture the 10kg batch (which may not be possible due to plant space limitations) or instead cycled a greater number of times per batch which will increase the process time. As a result chromatography effectively imposes a constraint upon the maximum productivity of a biopharmaceutical manufacturing facility. Given the present market for mAb therapeutics, cases in which these constraints have been reached are rare (see Kelley B. Very Large Scale Monoclonal Antibody Purification: The Case for Conventional Unit Operation. Biotechnology Progress 2008; 23: 995-1008). However, in the face of increasing product demand, some engineers have questioned the long term sustainability of the current paradigm for mAb purification. Companies now have an ever increasing portfolio of mAb candidates in the development pipeline. Even if only a fraction of these candidates make it through clinical trials, this will still lead to the need for multi-product facilities, operating very quick, intensive manufacturing campaigns which may challenge productivity constraints. Additionally, mAbs products are finding applications beyond those for which they were originally intended. This could dramatically increase the demand for a particular mAb which may again push plant productivities beyond their limit.

In light of this, process engineers have begun investigating what may be termed alternative bioseparation technologies, which have the potential to offer higher processing capacities, as well as possibly providing better economies of scale than packed bed chromatography (see Przybycien, T. M.; Pujar, N. S.; Steele, L. M. Alternative bioseparation operations: life beyond packed-bed

chromatography Current Opinion in Biotechnology 2004, 15 (5), 469-478). Two such separation techniques are aqueous two phase extraction (ATPE) and precipitation. These have attracted interest due to their relatively low associated operating costs and ease of scaling. Also because both are based on bulk mixing of the process fluid, these techniques will scale with the volume of the process stream, rather than the mass of product contained within, as is the case with packed bed chromatography. For example a 10,000L cell culture would require a 10,000L precipitation tank, regardless of whether the cell culture produced mAb at a concentration of 1g/L or 10g/L. A drawback of precipitation processes is the relatively low purification factors achievable. This is down to not only the non-specific mechanism of separation, but also the potential for impurity entrapment within the precipitate complex, leading to the need for extensive precipitate washing prior to resolubilisation in order to maximise product purity. Process robustness is another issue, with screening of a wide range of conditions necessary to determine the optimal operating parameters for each new antibody product.

Likewise aqueous two phase extraction (ATPE) has also been found to suffer some drawbacks. Several studies have been performed in which aqueous two phase extraction processes have been optimised for the purification of monoclonal antibodies (see Andrews BA, Nielsen S, Asenjo JA. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems, Bioseparation 1996; 6: 303-313. and Azevedo AM, Rosa PAJ, Ferreira IF, Aires-Barros MR. Optimisation of aqueous two-phase extraction of human antibodies, Journal of Biotechnology 2007; 132: 209-217). These studies showed relatively promising results, with high antibody yields achieved, however only modest purification factors were obtained due to the non-specific mechanism of separation employed by ATPE processes.

Key processing parameters associated with ATPE are all concerned with affecting the partitioning behaviour of the process stream components. Whether a molecule partitions into the top or bottom phase will be determined by the properties of the molecules (e.g. charge, MW and solvation) as well as those of the polymer (e.g. conc., MW, hydrophobicity). The physicochemical environment (e.g. temperature, pH and ionic strength) of the system will also influence partitioning behaviour. Due to the various interacting factors and the careful balance required between different operating parameters to ensure optimal performance, ATPE systems can be relatively non-robust. The situation is further exacerbated by the lack of fundamental knowledge regarding the partitioning of biological components in aqueous two phase systems. Process optimisation therefore requires a screening of a wide range of conditions along with the adoption of a design of experiments approach. As a result, for a new antibody product, extensive process development of an ATPE operation is not only a very necessary endeavour, but it is also a lengthy one. This compares unfavourably with Protein A affinity chromatography which displays a high level of robustness and only requires fine tuning of operating conditions in order to achieve optimal performance for a new antibody product.

The partitioning behaviour of different aqueous two phase systems, in terms of phase volume

ratios, can be affected not only by the choice of phase forming components and their concentration within the system, but also upon the properties of the feed material. Product concentration is a key aim for any bioseparation technique to be employed early on in a downstream process. Skewed phase volume ratios can adversely affect the concentrating power of ATPE since antibodies may partition to the high volume phase. Recently several patent applications have been published, regarding the use of precipitation for the purification of protein therapeutics. These are:

- Coffman et al. Separation Methods (US 2007/0066806);
- Farhner et al. Polyelectrolyte Precipitation and Purification of Proteins (US 2008/0193981);
- Ramanan et al. Method of Isolating Antibodies by Precipitation (WO 2008/100578); and
- Moya et al. Purification of Proteins (WO 2008/079302).

Whilst all aforementioned patent applications are concerned with the purification of monoclonal antibodies (mAb), there are differences in the specific route through which this purification is achieved. WO 2008/079302 and US 2007/0066806 are both concerned with precipitation of impurities in the process stream rather than the target antibody. Removal of the precipitated impurities then allows for purification of the mAb. The precipitation processes described by WO 2008/100578 and US 2008/0193981 can be applied to the precipitation of either target antibody product or impurities in the process stream.

The precipitation processes described by these applications can all be used to process crude fermentation broth containing whole cells. The methods described in WO 2008/100578, US 2008/0193981 and WO 2008/079302 may only be used in this manner if the component(s) being precipitated are process stream impurities. The precipitation process described by US 2007/0066806 can only be used to precipitate impurities. These applications describe methods which utilise different precipitating agents. US 2008/0193981 describes a method of precipitation which utilises polyelectrolytes that can interact with the target molecule allowing for selective precipitation. US 2007/0066806 utilises a combination of soluble salts which react with one another, when in solution together, to form insoluble salt precipitates. These precipitates associate (during and after formation) with impurities in the process stream allowing for selective precipitation. WO 2008/100578 utilises soluble polymers which have an affinity for the target molecule. Introduction of physicochemical stimuli (e.g. changes in temperature, pH, ionic strength etc.) can cause this polymer to come out of solution and form a precipitate, bringing the target molecule with it. WO 2008/079302 utilises a combination of isoelectric precipitation and also PEG as an additional precipitant. The process must also be performed at relatively low temperatures (2-8^{o}C) in order to reduce further the solubility of the target molecule.

There remains a need for a separation technique which is considerably more robust and which achieves comparable performance across significantly different feed materials containing different therapeutic proteins of interest.

12.5 Summary of the Invention

A process for the purification of proteins is provided. This process is based on a polymer - salt Aqueous Two Phase Extraction (ATPE) performed on a multi-component mixture (i.e., the feed) containing the target protein, ultimately resulting in precipitation of the product which may be re-solubilised and passed on for further purification. The novel ATPE - precipitation process is comprised of two discrete stages. The first forward extraction stage involves introducing the phase forming components (such as Polyethylene Glycol (PEG), Phosphate and NaCl) to the feed causing the formation of a polymer-salt aqueous two phase system in which the target protein preferentially partitions to the polymer rich phase, whilst some impurities move either into the salt rich phase or collect in the form of a precipitate at the interface between the two phases. In the second back extraction stage, the polymer rich phase from the forward extraction is recovered and contacted with a back extraction buffer (for example a phosphate buffer), forming a second aqueous two phase system and in turn causing the target protein to move out of the polymer rich phase and collecting in the form of an interfacial precipitate. This product containing precipitate is recovered using a filter or a centrifuge and then re-solubilised in an appropriate re-solubilisation buffer allowing for further processing. In cases which the process was used for the primary capture of monoclonal antibodies (mAbs) from mammalian cell culture supernatants, yields from this ATPE assisted precipitation process were found to be comparable to yields obtained using protein A chromatography, across a range of different mAbs and feeds. This process thus offers a potential alternative for the primary capture of mAbs from cell culture supernatant. This method overcomes the drawbacks associated with precipitation and ATPE processes individually by integrating a precipitation process with a two-stage ATPE process. The first forward extraction stage of the ATPE process, allows partial purification of the product mAb, as it preferentially partitions to the polymer rich phase. The second back extraction process, performed on the polymer rich phase recovered from the forward extraction allows for further purification as impurities partition either to the top or the bottom phase, whilst the product mAb precipitates at the interface between the two phases. A combination of purification mechanisms allows for a product which is not only purer than that which may typically be obtained with precipitation or ATPE individually, but also can be obtained in a much more concentrated form than that which might be obtained from a typical ATPE process alone. The combination of ATPE and precipitation has also yielded a separation technique which is considerably more robust, showing comparable performance across significantly different feed materials containing different target proteins.

12.6 Brief Description of the Drawings

As a means of illustration, Figures 12.1 - 12.11 refer to the use of the invention described herein, for the purification of a monoclonal antibody. Figure 12.1 shows the process scheme for a PEG - phosphate forward extraction aqueous two phase system at either preparative or manufacturing scale, being used to capture a mAb from a mammalian cell culture. Figure 12.2 shows the process scheme for a PEG phosphate back extraction aqueous two phase system at either preparative or manufacturing scale, being used to capture a mAb from a mammalian cell culture. Figure 12.3 shows a process scheme for mAb precipitate recovery and resolubilisation at either preparative or manufacturing scale. Figure 12.4 shows an overall process flow sheet illustrating the possible equipment requirements for this ATPE augmented precipitation process at either preparative or manufacturing scale. Figure 12.5 is a schematic showing a monoclonal antibody purification process flow incorporating certain embodiments of the invention. This diagram serves to illustrate the ways in which the method described may be employed from a whole bioprocess perspective. Figure 12.6 shows a comparison of Chromatograms from Protein A analyses of top and bottom phases of a forward extraction aqueous two phase system applied to a cell culture feed supernatant containing antibody A and denoted cell culture supernatant feed A. Figure 12.7 shows a comparison of Chromatograms from Protein A analyses of top and bottom phases of a forward extraction aqueous two phase system applied to a cell culture feed supernatant containing antibody B and denoted cell culture supernatant feed B. Figure 12.8 shows a comparison of Chromatograms from Protein A analyses of top and bottom phases of a back extraction aqueous two phase system performed on the top phase obtained from the forward extraction on cell culture supernatant feed A. Figure 12.9 shows a comparison of Chromatograms, similar to Figure 8, of top and bottom phases of a back extraction aqueous two phase system performed on the top phase obtained from the forward extraction on cell culture supernatant feed B. Figure 12.10 shows a comparison of Chromatograms from Protein A analyses of the top phase obtained from the forward extraction performed on cell culture supernatant feed B, containing antibody B and of the resolubilised precipitate formed in and recovered from the back extraction aqueous two phase system. Figure 12.11 shows a comparison of Chromatograms from size exclusion chromatography analyses of the cell culture supernatant feed B, containing antibody B, the top phase from the forward extraction aqueous two phase system performed on cell culture supernatant feed B and the resolubilised precipitate subsequently formed in the back extraction aqueous two phase system.

12.7 Detailed Description of the Invention

12.7.1 Definitions

Terms used in the description of the invention are collected and defined. The term target molecule, target protein and protein product refers to the protein which it is the aim of the method, to cause precipitation of. The protein includes both therapeutic protein and antibody. The term multi-component mixture refers to any aqueous mixture containing more than one type of biological or organic molecule including, recombinant proteins, native host cell proteins, DNA, RNA, viruses and lipids. Aqueous mixtures encompassed by the term multi-component mixture, may also contain unlysed whole cells of various types including mammalian, microbial and yeast. The term also covers aqueous mixtures containing fragments of cells, resulting from the lysing and/or homogenisation of mammalian, microbial and yeast cells. The term multi-component mixture specifically encompasses mammalian cell culture supernatant and clarified microbial fermentation broth. The term also encompasses clarified microbial lysate and homogenate, blood and blood fractions, and partially purified variants of all multi-component mixtures which have been herein defined to be specifically encompassed by the term. The term antibody means any recombinant of naturally occurring intact antibody, e.g. an antibody comprising an antigen-binding variable region as well as a light chain constant domain (CL) and heavy chain constant domains. Also encompassed by the term are antibody fragments, or molecules including antibody fragments, including, but not limited to, Fab, Fab, F(ab)2, Fv and Fc fragments. The term antibody specifically encompasses fusion proteins such as Fc fusion proteins, peptibodies and other chimeric antibodies. The term antibody specifically encompasses both monoclonal and polyclonal antibodies. The term cell culture supernatant refers to cell culture media from which whole cells have been removed by, for example, filtration. Cell culture supernatant can be but need not be clarified. For the purpose of the invention, the cell culture supernatant is one form of a multi-component mixture which contains the target protein of interest. The term host cell proteins refers to all proteins expressed by the cultured host cell, aside from the protein product, during the course of the cell culture process. The term aforementioned patents refers to US 2007/0066806 (Coffman et al.), US 2008/0193981 (Farhner et al.), WO 2008/100578 (Ramanan et al.) and WO 2008/079302 (Moya et al.) The term aqueous two phase system refers to an aqueous mixture composed of two water-based immiscible aqueous solutions. The term incompatible anion refers to the anion of salts which when in solution with certain polymers, will cause the solution to separate, forming two discreet phases; a polymer rich phase and a salt rich phase. Anions encompassed by the term incompatible anion include kosmotropic anions such as phosphate (PO_4^{3-}), citrate ($C_3H_5O(COO)_3^{3-}$) and sulphate (SO_4^{2-}). The term phosphate unless the context clearly dictates otherwise, or it is explicitly stated otherwise, refers to a salt of phosphoric acid, for example sodium phosphate, rather than the phosphate ion (PO_4^{3-}). The term polymer rich phase,

refers to the phase of an aqueous two phase system, which contains the highest concentration of polymer or polymers. The term salt rich phase, refers to the phase of an aqueous two phase system, which contains the highest concentration of the incompatible anion of the salt used to form the two phase system. The term top phase, refers to the less dense phase of an aqueous two phase system which collects above the bottom phase and also any interfacial precipitate which may have formed in an aqueous two phase system when either the two phase system is left to settle under the influence of gravity, or if the settling is assisted, for example through the use of centrifugation. The term bottom phase refers to the more dense phase of an aqueous two phase system which collects below the top phase and also any interfacial precipitate which may have formed in an aqueous two phase system when either the two phase system is left to settle under the influence of gravity, or if the settling is assisted, for example through the use of centrifugation The term interfacial precipitate refers to precipitate which forms in an aqueous two phase system and which collects at the interface between the top and bottom phases when either the two phase system is left to settle under the influence of gravity, or if the settling is assisted, for example through the use of centrifugation.

12.7.2 Method for Purification of a Target Protein

The method provided herein is an integrated two step ATPE assisted precipitation process in which the first, so called forward extraction ATPE step removes impurities via preferential partitioning of said impurities to the salt rich phase of the two phase system, as well as causing the precipitation of some impurities, while the second, so called back extraction ATPE step precipitates the product. It is integrated because the conditions of the process stream following the forward extraction step directly prepare it for the second, back extraction step. In certain embodiments the target protein can be an antibody. The antibody can be either a monoclonal or polyclonal antibody. The antibody can also be an IgG antibody, for example an IgG1, IgG2, IgG3 or IgG4 antibody. Also encompassed by the term antibody are antibody fragments, chimeric antibodies, fusion proteins such as Fc fusion proteins and peptibodies. In other embodiments, the target protein could be a recombinant protein such as recombinant human growth hormone, recombinant human insulin or interferon. The target protein could also be an enzyme, either in recombinant or native form. In further embodiments the target protein could also be a blood factor. The purification process described herein can be applied to any multi-component mixture, in which the aim is to isolate and purify a protein product within the mixture from other components, which may include, but is not limited to, native host cell proteins, DNA, RNA, viruses and lipids. In one embodiment of this present invention, the multi-component mixture (i.e., the feed material of the purification process) is a cell culture supernatant, generated through the culture of mammalian cells expressing and secreting an antibody of interest into the culture media. The cell culture supernatant may be obtained by either filtration or centrifugation of

the cell culture broth, allowing for removal of whole cells. The feed to the purification process should therefore be preferably free of unlysed whole cells. The cell culture supernatant, however, need not be clarified. Feed material containing whole unlysed cells may be used, providing the composition and conditions of the ATPE forward extraction system cause the antibody product to preferentially partition into the top phase, since in such a system whole cells will move into the bottom phase, The feed material may also contain large cell fragments such as cell debris, which will either partition to the bottom phase or precipitate at the interface during the forward extraction. The optional clarification of the cell culture supernatant may be accomplished using any conveniently available method, for example microfiltration or depth filtration. The aqueous two phase extraction assisted precipitation purification method described herein is comprised of three discrete stages.

- 1. Forward extraction
- 2. Back extraction
- 3. Precipitate recovery and resolubilisation

1. Forward Extraction

The first forward extraction stage involves forming a polymer-salt aqueous two phase system in which conditions are such that the target protein (e.g., antibody) preferably partitions into the polymer rich phase. The two phase system may be formed by adding appropriate amounts of phase forming components, to the feed. Phase forming components should include at least one water soluble polymer, a soluble salt with an anion which, when in solution is incompatible with the polymer and therefore capable of forming an aqueous two phase system with it, and another soluble salt which is used to mediate the partitioning of components in the two phase system. The water soluble polymer may be selected from a list including but not limited to Polyethylene glycol (PEG) or ethylene oxide-propylene oxide (EOPO). The incompatible salt should contain a strongly hydrated anion, and may be selected from a list including but not limited to citrate, phosphate or sulphate. The partition mediating salt should contain a less hydrated anion and may be selected from a list including but not limited to chloride, iodide or nitrate. In one embodiment, the polymer is a polyethylene glycol (PEG). The PEG can have a molecular weight of between 1,450Da and 6,000Da, for example 1,500Da. The incompatible salt is a mixture of monobasic and dibasic sodium phosphate salt and the partitioning mediating salt is sodium chloride (NaCl). In another embodiment, the polymer is PEG with a molecular weight of 4,000Da, the incompatible salt is sodium citrate and the partition mediating salt is potassium iodide (KI) In one embodiment of the invention the phase forming components are added to the feed in the form of powders. In another embodiment the phase forming components are added to the feed in the form of concentrated stock solutions. In another embodiment, some of the phase forming components are added in the form of powders whilst others

are added in the form of concentrated stock solutions. For example in a PEG - Phosphate ATPE system, with NaCl as the partition mediating salt, the PEG and the phosphate may be added to the feed material in the form of concentrated stock solutions, whilst the NaCl is added in powder form. The phase forming components should be added in such relative quantities so as to cause the formation of an aqueous two phase system, within which components in the feed display the desired partitioning behaviour. Suitable system compositions may be found in the extensive study performed by Albertsson et al., (see Albertsson, P.-A, 1986. Partition of Cell Particles and Macromolecules, third edition. Wiley, N.Y.). One of ordinary skill in the art will recognise the need to optimise the relative concentration of the phase forming components to not only provide desirable partitioning behaviour during the forward extraction, but also so as to result in a polymer rich phase which is amenable to the back extraction process, and the precipitation of the product protein caused therein. In one embodiment PEG, phosphate and NaCl are added so as to result in a final system composition of 12% - 20% (w/w) PEG, 9% - 19% (w/w) Phosphate and 4% - 12% (w/w) NaCl. In another embodiment PEG, a mixture of monobasic and dibasic phosphate and NaCl are added so as to result in a final system composition of 15% (w/w) PEG, 14% (w/w) phosphate and 12% (w/w) NaCl. The monobasic phosphate added may be monobasic sodium phosphate (NaH_2PO_4) or monobasic potassium phosphate (KH_2PO_4). The dibasic phosphate used may be dibasic potassium phosphate (K_2HPO_4) or dibasic sodium phosphate (Na_2HPO_4). In determining the amount of phosphate salt to be added to such a system, the mass composition of phosphate should include not only the weight of the phosphate ion, but also the cation of the salt. For example in forming a system with a final overall mass of 30 grams, with the phosphate added in the form of a powder, 4.2g of anhydrous monobasic sodium phosphate (NaH_2PO_4) powder should be added, in order to give a final Phosphate concentration of 14% (w/w), even though the mass fraction of phosphate (PO_4^{3-}) in monobasic sodium phosphate (NaH_2PO_4) means that only 3.325g of phosphate has been added which corresponds to a PO_4^{3-} concentration of approximately 11.1% (w/w). Hydrated salt powders may be used, for example monobasic sodium phosphate monohydrate ($NaH_2PO_4.H_2O$), but the mass contribution of water must be accounted for. Similarly is the case for when adding phosphate in the form of a concentrated stock solution. The phase forming components should preferably be added sequentially, with each component allowed to fully dissolve (when added in the form of a powder) and/or disperse (when added in the form of a concentrated stock solution) under mixing of the bulk fluid, prior to the next component being added. The phase forming components may be added in any order. The components can all be added at once, however this may have undesirable results such as excessive precipitation and longer salt and polymer dissolution and/or dispersion times, caused by poor fluid mixing as a result of increased fluid viscosity. The dissolution/dispersion of phase forming components may be performed at room temperature and pressure, although this may be performed at any temperature which is found to be conducive towards the dissolution/dispersion of

phase forming components, for example some Polyethylene glycols are found to be more soluble at lower temperatures. The system pH should be at least 1 pH unit below the pI of the target protein or antibody in order to ensure the target protein is positively charged. This will not only insure against premature precipitation, but also help partitioning of the target protein to the polymer rich phase of the two phase system. In an embodiment of the invention in which a mixture of monobasic and dibasic phosphate is used, the system pH may be established by altering the ratio between the amount of monobasic and dibasic phosphate salt added to form the two phase system. Increasing the amount of monobasic phosphate used, whilst decreasing the amount of dibasic phosphate added will result in a lower system pH, while increasing the amount of dibasic phosphate added and decreasing the amount of monobasic phosphate used will increase the pH of the system. The pH of the forward extraction system may be between pH 3.0 and pH 9.0, although a relatively neutral pH is preferable, for example pH 5.0 to 7.0, such as pH 6.0. Following complete dissolution and/or dispersion of the phase forming components, the forward extraction system should be left to mix for between 10 minutes and 1 hour, for example 30 minutes, before being incubated at room temperature for between 10 minutes to 24 hours, for example 30 minutes. The mixing period following complete powder dissolution and/or stock solution dispersion, is to firstly maximise the surface area for mass transfer between phases, and also to ensure equilibrium is reached with regards to partitioning of components between the polymer rich phase and the salt rich phase. The incubation period is to allow for phase separation under gravity. Dependent upon the complexity of the feed, the presence of precipitate and hence the resultant viscosity of the two phase system, complete phase separation may be accomplished in this manner. In an embodiment in which the present invention is used to purify an antibody product from a cell culture supernatant, the complexity of the cell culture supernatant will mean that this eventuality is unlikely due to an increase in system viscosity, caused by the precipitation of feed components during the forward extraction. Instead complete phase separation and recovery of the polymer rich phase may be accomplished using any conveniently available method, for example centrifugation. Any precipitate formed during the forward extraction, which will settle (possibly requiring some assistance, for example using centrifugation) at the interface between the top and bottom phase. This precipitate will be composed mostly of impurities and contain negligible amounts of target protein and as such its recovery is not required. In the forward extraction, a combination between salting out effects and electrostatic interactions will cause the target protein to preferably partition into the polymer rich phase. The partition coefficient (top phase concentration/ bottom phase concentration), denoted K, of the target protein should be between 5 and >100, for example K=50. In one embodiment of this invention, the forward extraction process may be performed in a single stirred vessel. The multi-component mixture (e.g., cell culture supernatant) may be held in an agitated vessel to which the phase forming components, PEG, phosphate and NaCl in powder form, are directly and sequentially added. The contents of this vessel may be mixed until all

phase forming components are completely dissolved. Further mixing may be performed in order to ensure equilibrium is reached and that complete partitioning of components has occurred. One of ordinary skill in the art will recognise the need to optimise the mixing process, in order to minimise mixing times within the stirred vessel. Optimisation of the mixing process will need to account for, among other things, factors such as the design of agitator(s), vessel dimensions and presence of baffles. Following on, once the mixing stage of the forward extraction process has been completed, agitation of the stirred vessel may be halted, and the contents allowed to settle under the influence of gravity. Dependent upon the viscosity of the aqueous two phase system and the time permitted for the incubation period, complete phase separation may be achieved in this manner. If so, the bottom portion of the vessel contents may be drained in order to remove the bottom phase from the system, leaving only the top phase, and any precipitate which may have formed during the forward extraction process, in the vessel. If the bottom phase corresponds to the polymer rich phase, then it may be clarified using any conveniently available method such as filtration or centrifugation in order to remove any precipitate carried over from the forward extraction process, before being passed onto the back extraction process. Alternatively, the top phase may be the polymer rich and therefore product containing phase. In such cases the top phase may be recovered using any conveniently available method, for example filtration or centrifugation. This embodiment is exemplified in Figure 12.1.

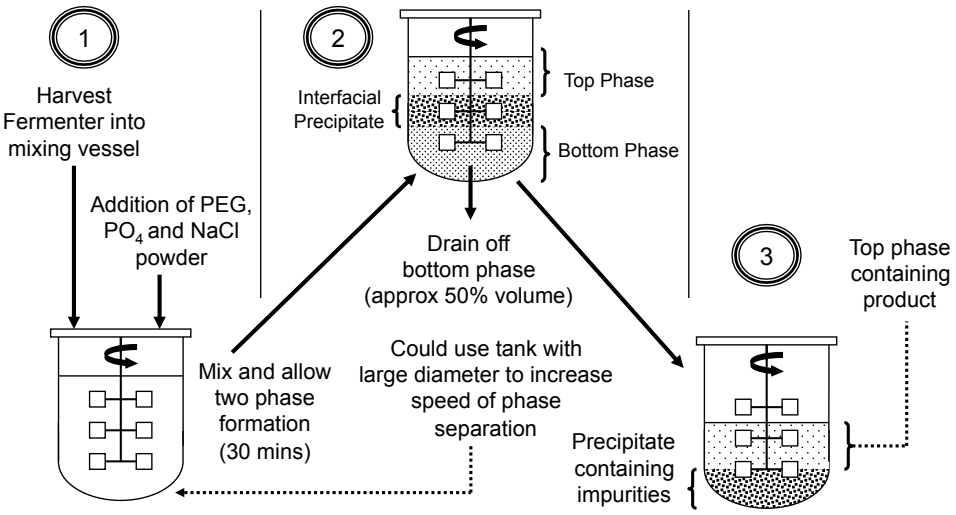


Figure 12.1

If phase separation is only partial under gravity, only a fraction of the bottom phase may be drained from the vessel. The remaining portion of bottom phase and also any precipitate which may have formed during the forward extraction process must be removed from the polymer rich top phase, using a conveniently available method, for example centrifugation. The recovered polymer rich, target protein containing top phase may then be passed on to the back extraction process.

2. Back Extraction

The back extraction step is performed following the forward extraction (Figure 12.2).

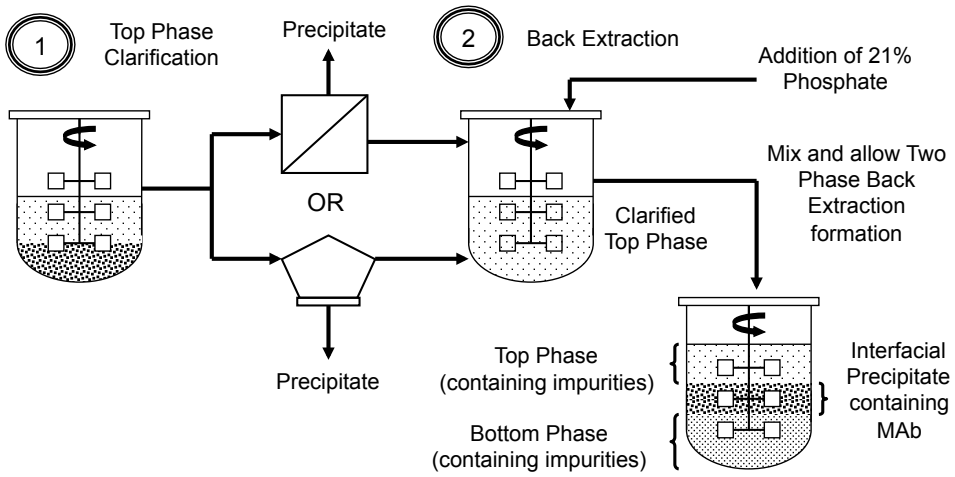


Figure 12.2

The polymer rich phase from the forward extraction is recovered and contacted with a back extraction buffer. The back extraction buffer can be a concentrated salt solution, containing a salt with an anion which is incompatible with the polymer used in the forward extraction system. The anion need not be the same as that which was used in the forward extraction system. In one embodiment the back extraction buffer is a phosphate salt solution with a concentration of between 10% (w/w) phosphate and 40% (w/w) phosphate, for example 21% (w/w) phosphate. The back extraction buffer is made using a combination of both monobasic and dibasic phosphate. The monobasic phosphate used may be monobasic sodium phosphate (NaH_2PO_4) or monobasic potassium phosphate (KH_2PO_4). The dibasic phosphate used may be dibasic potassium phosphate (K_2HPO_4) or dibasic sodium phosphate (Na_2HPO_4). The ratio of monobasic to dibasic phosphate added may

be altered to control the pH of the back extraction buffer. Increasing the amount of monobasic phosphate used, whilst decreasing the amount of dibasic phosphate added will result in a lower system pH, while increasing the amount of dibasic phosphate added and decreasing the amount of monobasic phosphate used will increase the pH of the system. The back extraction buffer should have a pH of between 3.0 and 9.0, although a relatively neutral pH is preferable, for example between pH 5.0 and 7.0, such as pH 6. In another embodiment, the back extraction buffer is a citrate salt solution with a concentration of between 10% (w/w) citrate and 40% (w/w) citrate, for example 30% citrate. This citrate back extraction buffer can be made using sodium citrate salt. The back extraction buffer should be mixed with the polymer rich phase from the forward extraction to form a new aqueous two phase system. The volume of back extraction buffer added should be between one and two times the volume of the polymer rich phase from the forward extraction. For example 10mL of back extraction buffer should be added to 10mL of polymer rich phase or 15mL of back extraction buffer should be added to 10mL of polymer rich phase or 20mL of back extraction buffer should be added to 10mL of top phase One of ordinary skill in the art will recognise the need to optimise both the concentration of the back extraction buffer and also the volume ratio between the polymer rich phase from the forward extraction and the back extraction buffer. The back extraction two phase system should be mixed for between 5 minutes and 30 minutes, for example 10 minutes. The aqueous two phase system may be left to incubate at room temperature for between 5 minutes and 60 minutes, for example 10 minutes. The mixing period is to maximise the surface area for mass transfer between phases, and also to ensure equilibrium is reached with regards to partitioning of components between the polymer rich phase and the salt rich phase. The incubation period is to allow for partial phase separation under gravity. Precipitation will occur during this back extraction process, which will settle (possibly requiring some assistance, for example using centrifugation) at the interface between the top and bottom phase. This precipitate will contain the majority of the target protein. Negligible amounts of the target protein will be present in the top and bottom phases of the back extraction system. In one embodiment the back extraction process may be performed in a single agitated vessel. Following clarification of the polymer rich phase from the forward extraction to remove any precipitate which may have been present, the polymer rich phase may be held in a stirred tank to which the back extraction buffer is directly added. The back extraction aqueous two phase system may then be mixed in order to ensure equilibrium is reached and that complete partitioning of components has occurred. One of ordinary skill in the art will recognise the need to optimise the mixing process, in order to minimise mixing times within the stirred vessel. Optimisation of the mixing process will need to account for, among other things, factors such as the design of agitator(s), vessel dimensions and presence of baffles. In addition, since the target protein forms a precipitate during this stage of the process, optimisation of the mixing process must account for the need to maintain precipitate integrity and also target product quality. Once mixing has been completed,

agitation of the stirred vessel may be halted, and the contents allowed to partially settle under the influence of gravity. The vessel will at this point contain a top and bottom phase containing the majority of impurities and an interfacial precipitate containing the majority of product protein. The next stage is to recover the precipitate formed during this back extraction process. This embodiment is exemplified in Figure 12.2.

3. Precipitate Recovery and Resolubilisation

The precipitate formed during the back extraction process may be recovered by any conveniently available method, for example microfiltration or centrifugation. One skilled in the art will recognise the need to optimise the precipitate recovery process depending upon the method employed. For example the use of a filtration will require optimisation of membrane surface areas and membrane fluxes in order to minimise the rate of membrane fouling and maximise process productivity. The use of centrifugation will require optimisation in order to maximise dewatering of the precipitate whilst also minimising precipitate compaction which may reduce the ease of resuspension. Regardless, trade-offs will need to be made between desirable process attributes. Following recovery, the precipitate may then also be washed using a suitable buffer in order to remove any residual liquid from the back extraction two phase system. This wash step is optional, but may be accomplished by contacting excess volumes of wash buffer with the recovered precipitate and removing the wash buffer using any conveniently available method, for example filtration, centrifugation or simply decanting. The precipitate may then be resuspended in a suitable buffer. The choice of resuspension buffer will depend upon a number of factors such as the characteristics of the target protein being purified as well as the requirements of the bioseparation technique to be employed following the purification method described herein. The resuspension buffer should have a pH of between 3.0 and 9.0. For example the resuspension buffer could be 60mM sodium citrate at pH 3.4. The resuspension of the product protein precipitate should be performed within 20 hours of initial formation during the back extraction process. Preferably the resuspension process should be performed within 6 hours or less following initial precipitate formation. For example the resuspension of the precipitate should be performed within 1 hour after formation during the back extraction process. Following resolubilisation, the product containing solution may be conditioned to a suitable pH and ionic strength, filtered to maintain sterility and placed into storage. For example when 60mM sodium citrate buffer at pH 3.4 is used for resuspension, the resultant product containing solution may be titrated using 0.1M sodium hydroxide (NaOH) to a more neutral pH, such as pH 5.0, before being filtered using a 0.22 micron filter to remove any potential bacterial or viral contamination. This sterile filtered solution may then be stored at 4^{o}C for later use and/or further purification. In one embodiment, in which the product is an antibody, the precipitate from the back extraction process is recovered on the surface of a microfilter. The back extraction aqueous two phase system is pumped

through a 0.22 micron filter at a suitable flow rate. The antibody containing precipitate is captured on the surface of the filter membrane whilst the top and bottom phases pass through into the filtrate. The membrane may be optionally washed with a suitable buffer to remove any residual top and bottom phase. The membrane may then be washed with the resolubilisation buffer, for example 60mM sodium citrate at pH 3.4, in order to resolubilise the precipitate, causing the antibody to emerge in the filtrate, which can then be collected in a holding vessel. This filtrate may then be passed on for further processing. One of ordinary skill in the art will recognise the need to optimise the precipitate resolubilisation in order to maximise the concentration of the antibody product, maximise the antibody yield as well as minimise buffer consumption. This will include, among other things, optimising the fluid flow through the membrane and across the membrane surface, as well as the number of times a volume of resolubilisation buffer is re-circulated through the membrane. The collected filtrate can then be, for example held at pH 3.4 in order to effect virus inactivation, before being titrated up to pH 5.0 or 6.0 using 0.1M sodium hydroxide. This conditioned product pool may then be filtered again to ensure and maintain sterility before being stored at 4^oC, for later use and/or further purification. This embodiment is illustrated in Figure 12.3.

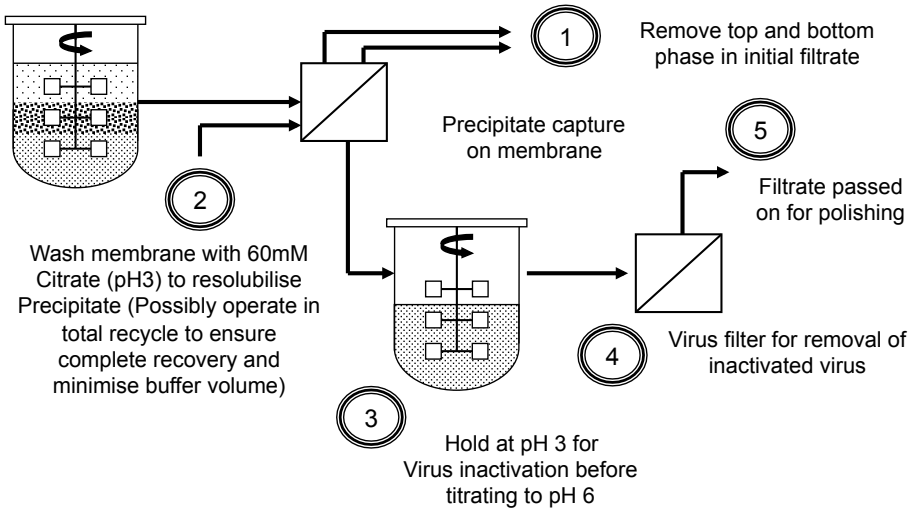


Figure 12.3

Figure 12.4 collects the embodiments illustrated by Figures 12.1 - 12.3 and shows the overall process flow sheet and the equipment requirements for this ATPE augmented precipitation process at preparative or manufacturing scale.

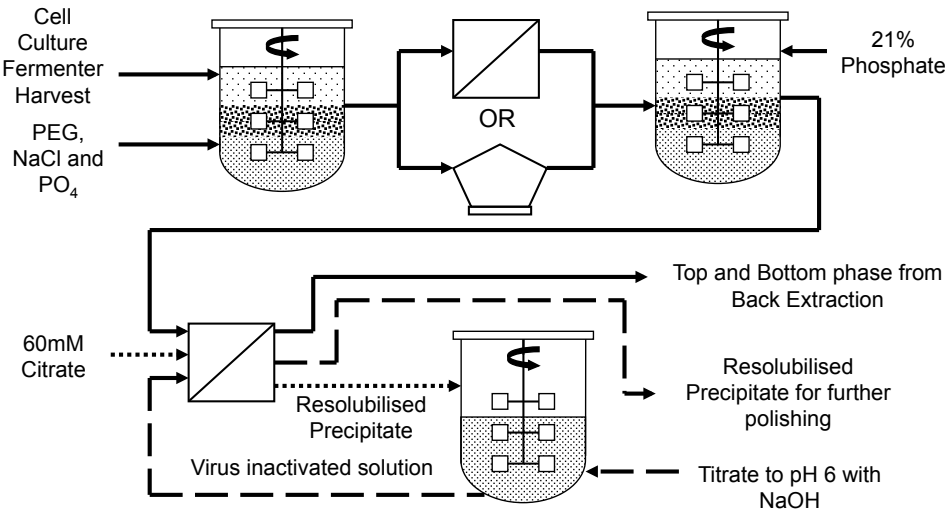


Figure 12.4

Overall three mixing vessels will be required in order to perform the forward extraction, back extraction and finally, if necessary, virus inactivation. The first vessel, used to perform the forward extraction may also be used to perform the virus inactivation provided that clean in place (CIP) and sterilise in place (SIP) procedures may be performed in time. In such a case the entire process may be performed using only two mixing vessels. Either a centrifuge or filtration unit will be required in order to recover the top phase from the forward extraction and then finally a filtration unit will be required in order to recover and resolubilise the precipitate and also for virus removal. A centrifuge may also be used to recover the product containing precipitate, which may then be transferred to a mixing vessel for resolubilisation. In one embodiment of this invention, the entire method may be employed in place of Protein A affinity chromatography for primary capture of antibody. The resolubilised precipitate may then be applied to unit operations which would be typically found subsequently after protein A affinity chromatography in a mAb purification process. For example the resolubilised precipitate may be run on a cation exchange chromatography column packed with for example Capto^{TM} S, in bind and elute mode. The antibody containing eluate from the cation exchange step may then be applied to an anion exchange column packed with for example Capto Q, in flow through mode with the antibody containing flow through collected. In another embodiment, the resolubilised precipitate containing the antibody may be applied to a multi-modal chromatography column packed with for example Capto adhere, in flow through mode. The antibody containing flow

through may then be applied to an anion exchange column packed with for example Capto Q, in flow through mode with the antibody containing flow through collected. These embodiments are collectively illustrated in Figure 12.5.

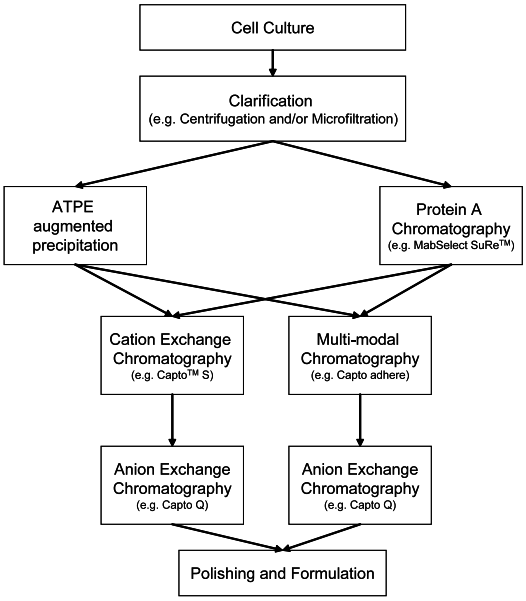


Figure 12.5

It is noted that all embodiments of this invention can be employed on any scale. For example, the present invention can be applied to large scale antibody production, in which the antibody is to be purified from tens of thousands of litres of cell culture supernatant. In another example the present invention may be employed on a much smaller scale, for example in bench top scale operations, in which antibodies are purified from several litres or less of cell culture supernatant.

12.8 Examples

The following examples serve to illustrate embodiments of the present invention. These examples are intended to demonstrate techniques which the present inventors have found to work well in practising the present invention. Hence these examples are detailed so as to provide those of ordinary skill in the art with a complete disclosure and description of the ways in which the methods of this invention may be performed. The following Examples are intended to be exemplary only and changes, modification and alterations can be employed to the conditions described herein, without departing from the scope of the invention.

12.8.1 Example 1 - Primary Capture and Purification of MAb using ATPE assisted precipitation

Chinese Hamster Ovary (CHO) cell culture supernatant was generated in house by GE Healthcare Biosciences (Uppsala, Sweden) through the culturing of cells from a cell line obtained from Polymun Scientific (Vienna, Austria). The cell culture supernatant was obtained by harvesting of the cell culture followed by centrifugation and depth filtration in order to remove whole unlysed cells. The cell culture supernatant was found to contain a monoclonal human IgG antibody, denoted Antibody A, at a titre of less than 1g/L. This supernatant was concentrated by approximately ten fold using ultrafiltration giving a final mAb concentration of 4.5g/L. This cell culture supernatant was sterile filtered using a 0.22 micron microfilter before being stored at 4°C prior to being subjected to the ATPE assisted precipitation process. Polyethylene glycol (PEG), with molecular weights of 1,500 and 6,000, along with Tris(hydroxymethyl)aminomethane and 3-bromopropyl trimethyl ammonium bromide were obtained in the form of powders from Sigma-Aldrich. Sodium phosphate monobasic monohydrate (NaH_2PO_4 . H_2O), potassium phosphate dibasic trihydrate (K_2HPO_4 . $3H_2O$), citric acid monohydrate ($C_6H_8O_7$. H_2O), tri-sodium citrate ($Na_3C_6H_5O_7$) and sodium hydroxide (NaOH) were also obtained in the form of powders and were purchased from Merck Chemicals Ltd (Nottingham, UK). Sodium chloride (NaCl) was obtained from VWR International Inc.

Aqueous Two Phase Extraction

I. Forward Extraction 30g ATPE forward extraction systems were generated by adding appropriate amounts of PEG 1500, K_2HPO_4 . $3H_2O$, NaH_2PO_4 . H_2O and NaCl powders directly to the cell culture supernatant feed so as to give a final system composition of 15% PEG 1500, 14% Phosphate and 12% NaCl. More specifically, 4.50g of PEG 1500, 2.44g of K_2HPO_4 . $3H_2O$, 2.66g of NaH_2PO_4 . H_2O and 3.60g of NaCl were added to 17.7mL of cell culture supernatant to form the ATPE forward extraction system. Systems were formed in 50mL Falcon tubes (BD Biosciences), with system mixing accomplished by placing the Falcon tubes onto a rocking platform shaker (custom manufactured at GE Healthcare). Powders were added sequentially with NaCl added first, followed by PEG 1500 and finally the K_2HPO_4 . $3H_2O$ and NaH_2PO_4 . H_2O. The monobasic and dibasic phosphates were added so as to give the desired system phosphate mass percentage whilst the pH of the system was controlled by altering the ratio between the mass of monobasic and dibasic salt added. In this case, the actual ratio between the amounts of monobasic and dibasic phosphate added, resulted in a forward extraction system with a pH of 6.0. Sufficient time was allowed between the additions of powders so as to ensure complete dissolution of the previously added component before the introduction of the next. Powders were usually completely dissolved after approximately 10 minutes. Precipitation was observed following addition of the phosphate salts Following addition and

dissolution of all powders systems were mixed for a further 60 minutes before being left to settle under gravity for 30 minutes in order to effect phase separation. Only partial phase separation was achieved, possibly due to the precipitate in the aqueous two phase system affecting the settling velocity of the bottom salt rich phase. Systems were centrifuged at 3000 rpm for 30 mins, using a Eppendorf 5810R (Eppendorf, Hamburg, Germany) centrifuge in order to obtain complete phase separation. The forward extraction system was observed to be composed of three discreet phases, settled one on top of the other, following centrifugation; a top polymer rich phase, an interfacial precipitate and a salt rich bottom phase. The top and bottom phases were then carefully separated and the volumes determined. Samples were taken and analysed in order to determine the concentration of antibody in each phase. Figure 12.6 is an example of the results obtained from this analysis. It shows a comparison of Chromatograms from Protein A analyses (using a MabSelect SuReTM 1mL HiTrapTM column) of top and bottom phases of a forward extraction aqueous two phase system applied to cell culture supernatant feed, containing antibody A and denoted cell culture supernatant feed A. The chromatogram for the Protein A analysis of this feed is also included for comparison.

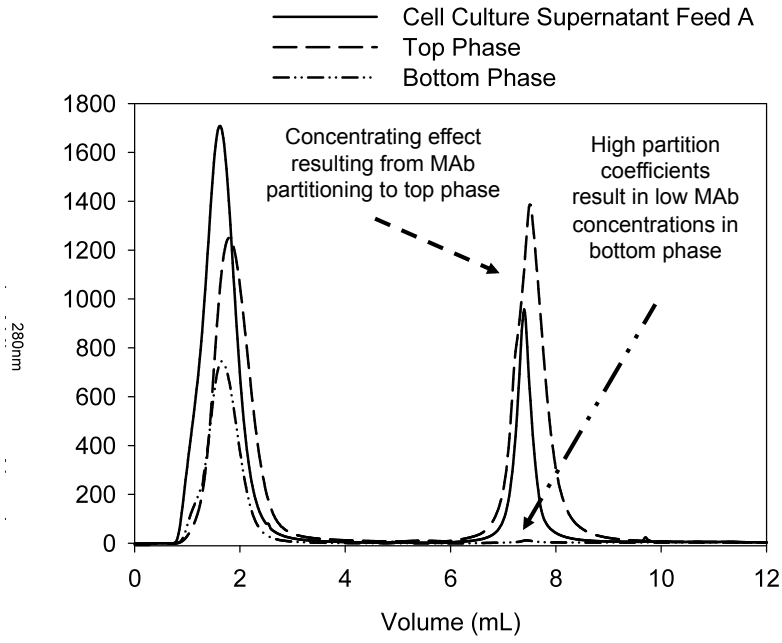


Figure 12.6

Antibody A is a monoclonal IgG. Peak 1, with a column retention of approximately 1.5 to 2mL in all chromatograms, corresponds to unbound UV280 adsorbing impurities present in the sample. Peak 2 with a retention of approximately 7.5mL in all chromatograms, corresponds to bound antibody A. The chromatograms shown in Figure 12.6 indicate a high level of antibody A partitioning to the

top phase of the forward extraction system with little to no antibody present in the bottom phase. Mass balances based on the integration of peaks shows partition coefficients of greater than 100. A mass balance also showed a yield of greater than 100% in the top phase. This indicates that the presence of PEG affects the UV absorbent properties of the antibody in some way (blank systems of PEG showed no absorbance at UV280nm). Due to this it is difficult to accurately determine the MAb content of the interfacial precipitate which was found to form during the forward extraction. Figure 12.7 shows a comparison of Chromatograms similar to that shown in Figure 12.6, albeit with a different feed material, this time a CHO cell culture feed supernatant, containing antibody B and denoted cell culture supernatant feed B.

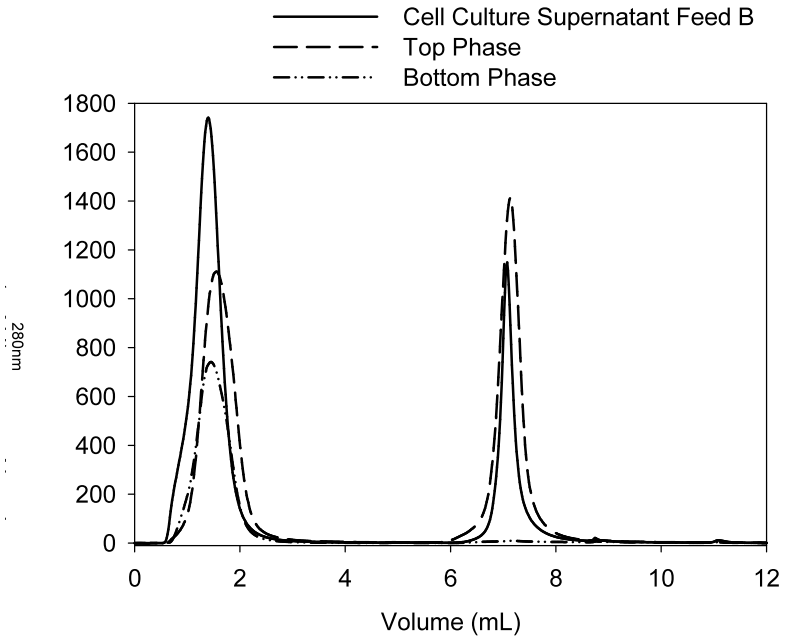


Figure 12.7

The chromatogram for the protein A analysis of this feed is included for comparison. Antibody B is also a monoclonal IgG. As with the results shown in Figure 12.6, Figure 12.7 shows a high level of antibody B partitioning to the top phase of the forward extraction system with little to no antibody present in the bottom phase. Mass balances based on the integration of peaks shows partition coefficients of approximately 70. Again mass balances showed a yield of greater than 100% in the top phase, apparently due to the presence of PEG affecting the UV absorbent properties of the antibody.

II. Back Extraction Back extraction was performed by taking the top phase from the forward extraction system and adding a back extraction buffer in order to generate a new two phase system.

The back extraction buffer utilised was a phosphate buffer solution, made using K2HPO4 . 3H2O and NaH2PO4 . H2O, added to give a phosphate concentration of 21% (w/w) and in a ratio as to give a pH of 6.0. Back extraction systems were formed in 50mL Falcon tubes, with back extraction buffer added to the top phase, recovered from the forward extraction, in a volume ratio (top phase : bottom phase) of 1:2. Back extraction systems were mixed in the same manner as the forward extraction systems, using the rocking platform, for approximately 10 minutes. Precipitation was observed during the mixing process. Back extraction systems were then allowed to settle under gravity for 15 minutes, before being centrifuged at 3000 rpm for 10 minutes to ensure complete phase separation using a Eppendorf 5810R (Eppendorf, Hamburg, Germany) centrifuge. The back extraction system was observed to be composed of three discrete phases, settled one on top of the other, following centrifugation; a top polymer rich phase, an interfacial precipitate and a salt rich bottom phase. Samples of the top and bottom phases were taken for analysis. Figures 12.8 and 12.9 show the chromatograms obtained from a Protein A affinity chromatography analysis of the top and bottom phases obtained from the back extraction systems.

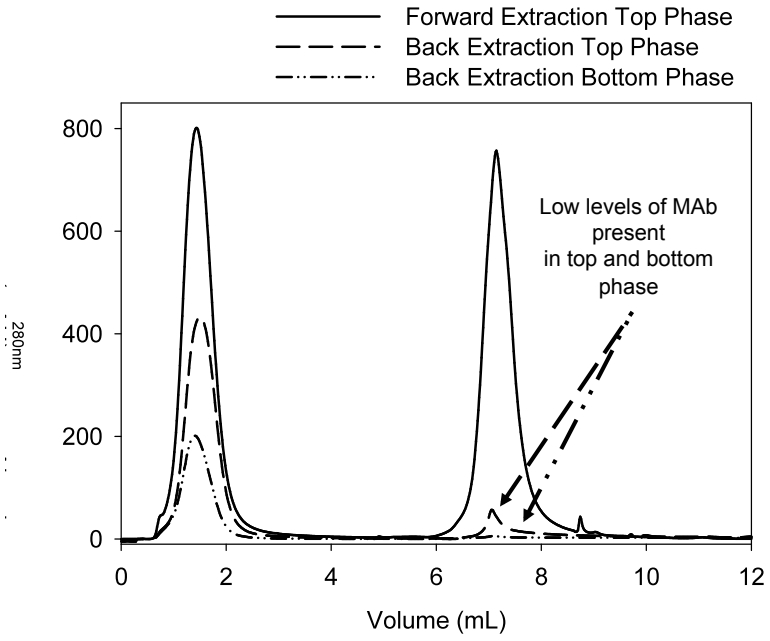


Figure 12.8

Figure 12.8 shows a comparison of Chromatograms from Protein A analyses of top and bottom phases of a back extraction aqueous two phase system performed on the top phase obtained from the forward extraction on cell culture supernatant feed A. The chromatogram from the Protein A analysis of the top phase from the forward extraction on CHO cell culture supernatant A containing

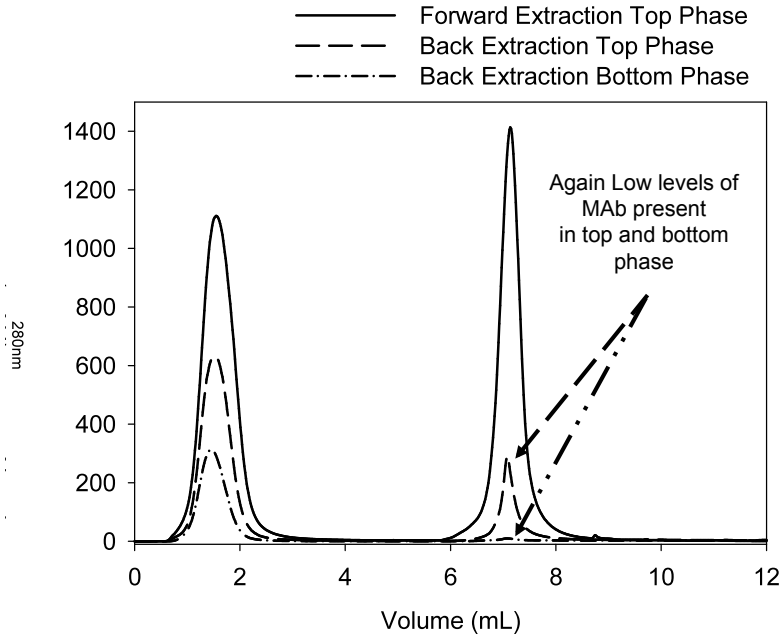


Figure 12.9

antibody A is also included for comparison. Peak 1, with a column retention of approximately 1.5 to 2mL in all chromatograms, corresponds to unbound UV280 adsorbing impurities present in the sample. Peak 2 with a retention of approximately 7.5mL in all chromatograms, corresponds to bound antibody A. The low concentrations of antibody A in top and bottom phase indicate that the majority of antibody has collected in the interfacial precipitate which was found to form during the back extraction. Based on antibody concentrations in top and bottom phase and that present in the top phase from the forward extraction, calculations indicate an antibody yield of between 85 and 90% in the precipitate. A mass balance using peak integration also indicates that this precipitate contains a low level of impurities. Figure 12.9 shows a comparison of Chromatograms, similar to Figure 12.8, of top and bottom phases of a back extraction aqueous two phase system performed on the top phase obtained from the forward extraction on cell culture supernatant feed B. The chromatogram from the Protein A analysis of the top phase from the forward extraction on CHO cell culture supernatant B containing antibody B is also included for comparison. Calculations indicate an antibody yield of between 85 and 90% in the precipitate. A mass balance using peak integration also indicates that this precipitate contains a low level of impurities.

Precipitate Recovery and Resolubilisation

The top and bottom phases of the back extraction system were carefully removed using a pipettor (VWR International Inc.) leaving only the precipitate in the Falcon tube. 60mM sodium citrate buffer at pH 3.4 was placed into the Falcon tube with the precipitate and mixed used a RX3 vortex mixer (VELP Scientifica, Italy). The precipitate resolubilised almost instantaneously upon mixing in this manner. Precipitate resolubilisation was performed at room temperature. This resolubilisation procedure was performed within 1 hour of initial precipitate formation during the back extraction process. After resolubilisation, the antibody containing sample was incubated at room temperature for 60 minutes for virus inactivation. The sample was then slowly titrated up to pH 5.0 using 0.1M NaOH. Samples were then analysed for antibody content, using Protein A chromatography. Figure 12.10 shows an example of the results obtained from this analysis. Specifically it shows a comparison of Chromatograms from Protein A analyses of the top phase obtained from the forward extraction performed on cell culture supernatant feed B, containing antibody B and of the resolubilised precipitate formed in and recovered from the back extraction aqueous two phase system. The chromatogram from the CHO cell culture feed supernatant, containing antibody B and denoted cell culture supernatant feed B is also included for comparison. Peak 1, with a column retention of approximately 1.5 to 2mL in all chromatograms, corresponds to unbound UV280 adsorbing impurities present in the sample. Peak 2 with a retention of approximately 7.5mL in all chromatograms, corresponds to bound antibody B.

Figure 12.10 shows the increase of MAb purity as it moves into the top phase during forward extraction and then into the precipitate during back extraction. The low concentration of MAb in the re-solubilised precipitate sample is due to the use of excess re-solubilisation buffer used in this particular experiment. The relative heights of the peaks indicate that this ATPE augmented precipitate process has afforded a significant level of purification. An analysis of the product quality and aggregate content throughout the ATPE augmented precipitation process was performed using size exclusion chromatography. As an example, Figure 12.11 shows a comparison of Chromatograms from size exclusion chromatography analyses (using a Superdex^{TM} 200 10/30 column) of the cell culture supernatant feed B, containing antibody B, the top phase from the forward extraction aqueous two phase system performed on cell culture supernatant feed B and the resolubilised precipitate subsequently formed in and recovered from the back extraction aqueous two phase system. The initial feed was found to have an aggregate content of approximately 16%. The aggregate content in the final re-solubilised precipitate was calculated to be approximately 20%. This is comparable to what would normally be achieved using Protein A as a primary capture step. The size exclusion analysis further indicates the significant level of purification achieved using this current process.

All patents, patent publications, and other published references mentioned herein are hereby incorporated by reference in their entireties as if each had been individually and specifically incor-

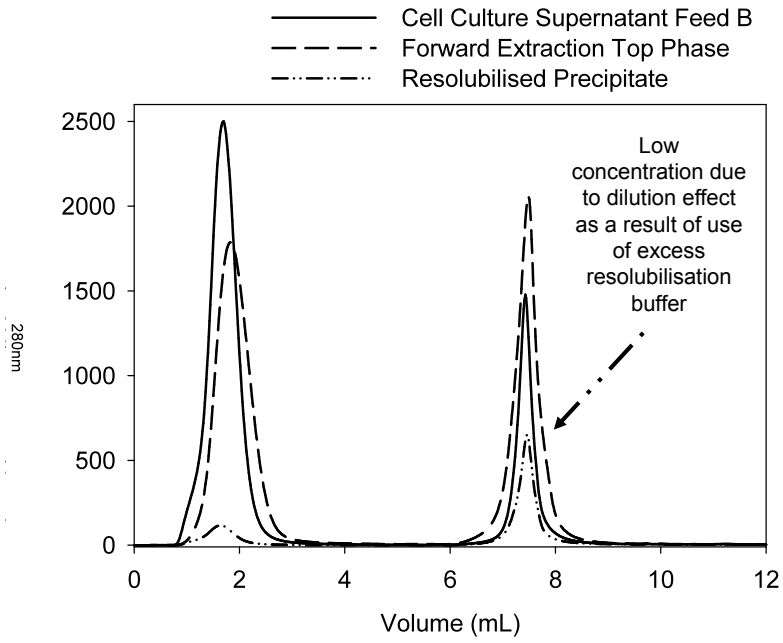


Figure 12.10

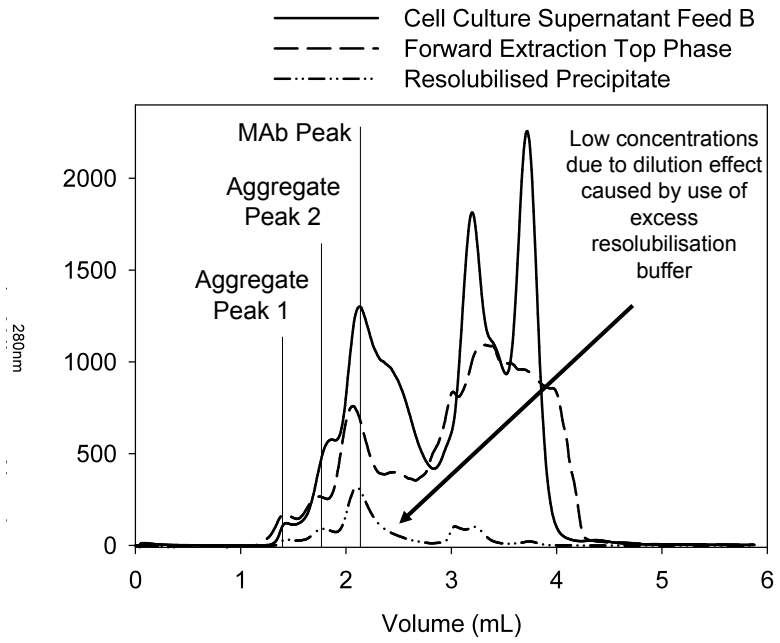


Figure 12.11

porated by reference herein. While preferred illustrative embodiments of the present invention are described, one skilled in the art will appreciate that the present invention can be practiced by other than the described embodiments, which are presented for purposes of illustration only and not by way of limitation. The present invention is limited only by the claims that follow.

12.9 References Cited

Patents and Patent Applications

- Coffman et al. Separation Methods (US 2007/0066806)
- Farhner et al. Polyelectrolyte Precipitation and Purification of Proteins (US 2008/ 0193981)
- Ramanan et al. Method of Isolating Antibodies by Precipitation (WO 2008/100578)
- Moya et al. Purification of Proteins (WO 2008/079302)

Other Publications

1. Albertsson, P.-A., 1986. Partition of Cell Particles and Macromolecules, third edition. Wiley, N.Y.
2. Andrews B.A., Nielsen S., Asenjo J.A. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. Bioseparation 1996; 6: 303-313.
3. Azevedo A.M., Rosa P.A.J., Ferreira I.F., Aires-Barros M.R. Optimisation of aqueous two-phase extraction of human antibodies. Journal of Biotechnology 2007; 132: 209-217.
4. Jacobi A., Eckermann C., Ambrosius D., Bioseparation and Bioprocessing 2nd Edition Volume 2 2007 Wiley-VCH pp 431-433.
5. Kelley B. Very Large Scale Monoclonal Antibody Purification: The Case for Conventional Unit Operation. Biotechnology Progress 2008; 23: 995-1008.
6. Przybycien, T. M.; Pujar, N. S.; Steele, L. M. Alternative bioseparation operations: life beyond packed-bed chromatography Current Opinion in Biotechnology 2004, 15 (5), 469-478.
7. Shukla A.A., Hubbard B., Tressel T., Guhan S., Low D. Downstream processing of monoclonal antibodies - Application of platform approaches. Journal of Chromatography B 848: 28-39.
8. Sommerfeld S. and Strube J. Challenges in biotechnology production - generic processes and process optimisation for monoclonal antibodies. Chemical Engineering and Processing 2005; 44: 1123-1137.
9. Thmmes J. and Etzel M. Alternatives to Chromatographic Separations. Biotechnology Progress 2007; 23: 42-45.

12.10 Claims

1. A method of recovering and purifying a protein from a multi-component mixture, the method comprising of:

   a. Adding phase forming components including a polymer, a salt containing an incompatible anion and a partition mediating salt to said multi-component mixture;

   b. Mixing and completely dissolving aforementioned phase forming components to form a forward extraction aqueous two phase system;

   c. Recovering the polymer rich phase;

   d. Contacting said polymer rich phase with back extraction buffer to form a back extraction aqueous two phase system, including an interfacial precipitate containing the protein;

   e. Recovering the interfacial precipitate from the two phase system; and

   f. Optionally, resuspending the precipitate in a resuspension buffer.

2. The method of claim 1, wherein the polymer is selected from Polyethylene glycol (PEG) and ethylene oxide-propylene oxide (EOPO), the incompatible anion is selected from strongly hydrated anions including phosphate, citrate and sulphate and the partition mediating salt is selected from less strongly hydrated anions, including NaCl and potassium iodide (KI)

3. The method of claim 2, wherein the polymer used is polyethylene glycol (PEG), the incompatible anion is phosphate and the partition mediating salt is NaCl.

4. The method of claim 3, wherein the concentration of PEG in the two phase system is between 12% and 20% (w/w), the concentration of Phosphate is between 9% and 19% (w/w) and the concentration of NaCl in the forward extraction aqueous two phase system is between 4% and 12% (w/w).

5. The method of claim 4, wherein the concentration of PEG in the forward extraction system is 15% (w/w), the concentration of phosphate is 14% (w/w) and the concentration of NaCl is 12% (w/w).

6. The method of claim 3, wherein the PEG added to the feed has a molecular weight of between 1,450Da and 6,000Da, such as a molecular weight of 1,500Da.

7. The method of claim 3, wherein the phosphate is added in the form of a mixture of monobasic and dibasic phosphate salts.

8. The method of claim 7, wherein the monobasic Phosphate is selected from monobasic sodium phosphate and monobasic potassium phosphate, and the dibasic phosphate is selected from dibasic sodium phosphate and dibasic potassium phosphate.

9. The method of claim 1, step (a), wherein the phase forming components are added to the feed in the form of powders.

10. The method of claim 1, wherein the pH of the forward extraction aqueous two phase system is between 3.0 and pH 9.0, such as a system pH of 6.0.

11. The method of claim 1, wherein following complete dissolution of phase forming components, the forward extraction system is incubated for between 10 minutes and 24 hours, such as for 30 minutes.

12. The method of claim 1, wherein some impurities, including viruses, are collected in the precipitate formed during the forward extraction or partition to the salt rich phase of the forward extraction system.

13. The method of claim 1, wherein the polymer rich phase of step (c) is recovered by:

    a. Gravity settling of the two phase system allowing for complete phase separation; followed by

    b. Draining of bottom phase with or without any interfacial precipitate or aspirating the top phase.

14. The method of claim 1, wherein the polymer rich phase of step (c) is recovered by:

    a. Centrifugation of the two phase system; followed by

    b. Removing the bottom phase and any interfacial precipitate or aspirating the top phase.

15. The method of claim 1, wherein the back extraction buffer is a concentrated salt solution.

16. The method of claim 15, wherein the anion of the salt composing the back extraction buffer is selected from citrate, phosphate and sulphate.

17. The method of claim 16, wherein the back extraction buffer is phosphate solution, with a concentration of between 10% (w/w) and 40% (w/w).

18. The method of claim 17, wherein the back extraction buffer has a pH of between 3.0 and 9.0, such as a pH of 6.0.

19. The method of claim 1, wherein the volume of back extraction buffer contacted with the polymer rich phase from the forward extraction is between one and two times the volume of the polymer rich phase from the forward extraction.

20. The method of claim 1, wherein following mixing, the back extraction aqueous two phase system is incubated for between 5 minutes and 15 minutes, such as for 10 minutes.

21. The method of claim 1, wherein the precipitate formed during the back extraction is recovered and resuspended by:

    a. Filtration of the back extraction system;

    b. Capturing the precipitate on the membrane surface; and

    c. Flushing the membrane with resuspension buffer to resuspend the antibody precipitate and collecting the filtrate.

22. The method of claim 21, wherein the resuspension buffer is re-circulated across and through the membrane to affect precipitate resolubilisation.

23. The method of claim 1, wherein the precipitate formed during the back extraction is recovered and resolubilised by:
    a. Centrifugation of the back extraction aqueous two phase system;
    b. Removal of liquid top and bottom phases; and
    c. Resuspending the precipitate in resuspension buffer.

24. The method of claim 1, wherein the resuspension buffer has a pH between 3.0 and 9.0

25. The method of claim 23, wherein the resuspension buffer contains 60mM sodium citrate at pH 3.4

26. The method of claim 1, wherein the method is carried out at room temperature and atmospheric pressure.

27. The method of claim 1, wherein the recovery and resuspension of the precipitate is performed within 20 hours, such as within 6 hours or within 1 hour, of formation during the back extraction process.

28. The method of claim 1, wherein the protein is a monoclonal antibody.

29. The method of claim 1, wherein the multi-component mixture is an unclarified cell culture or a cell culture supernatant.

Chapter 13

Appendix C: Published Chapters

Chapter 5

R Tran, Y Zhou, K M Lacki, N J Titchener-Hooker. A Methodology for the Comparative Evaluation of Alternative Bioseparation Technologies. *Biotechnology Progress*, 24 (2): 1007-1025, 2008.

Bibliography

[1] A Jacobi, C Eckermann, and D Ambrosius. *Bioseparation and Bioprocessing 2nd Edition*, volume 2, chapter Developing an Antibody Purification Process, pages 431–457. Wiley-VCH, 2008.

[2] J Reichert and A Pavlou. Monoclonal antibodies market. *Nature Reviews*, 3:383–384, 2004.

[3] S Lawrence. Billion dollar babies - biotech drugs as blockbusters. *Nature Biotechnology*, 25(4):380–382, 2007.

[4] G Walsh. Modern antibody-based therapeutics. *BioPharm International*, December 2004.

[5] S S Farid. Process economics of industrial monoclonal antibody manufacture. *Journal of Chromatography B*, 848:8–18, 2007.

[6] E E Hood, S L Woodard, and M E Horn. Monoclonal antibody manufacturing in transgenic plants - myths and realities. *Current Opinion in Biotechnology*, 13:630–635, 2002.

[7] J Birch. Future of antibody manufacturing. Bioproduction Conference, Amsterdam October 24-27, 2005.

[8] K A Thiel. Biomanufacturing, from bust to boom...to bubble? *Nature Biotechnology*, 22:1365–1372, 2004.

[9] B Kelley. Very large scale monoclonal antibody purification The case for conventional unit operations. *Biotechnology Progress*, 23:995–1008, 2008.

[10] A A Shukla, B Hubbard, T Tressel, S Guhan, and D Low. Downstream processing of monoclonal antibodies - application of platform approaches. *Journal of Chromatography B*, 848:28–39, 2007.

[11] Thömmes. Alternatives to chromatographic separations. *Biotechnology Progress*, 23:42–45, 2007.

[12] R Hahn, R Schlegel, and A Jungbauer. Comparison of protein A affinity sorbents. *Journal of Chromatography B*, 790:35–51, 2003.

[13] J Sjöquist, B Meloun, and H Hjelm. Protein a isolated from staphylococcus-aureus after digestion with lysostaphin. *European Journal of Biochemistry*, 29(3):572, 1972.

[14] R Lindmark, J Movitz, and J Sjöquist. Extracellular protein-a from a methicillin-resistant strain of staphylococcus-aureus. *European Journal of Biochemistry*, 74(3):623–628, 1977.

[15] H Engel, H Mottl, and W Keck. A modified vector for the controlled high-level overproduction of staphylococcal protein-a fusion proteins in the periplasm of escherichia-coli. *Protein Expression and Purification*, 3(2):108–113, 1992.

[16] P A J Rosa, A M Azevedo, S Sommerfeld, W Backer, and M R Aires-Barros. Aqueous two-phase extraction as a platform in the biomanufacturing industry: Economical and environmental sustainability. *Biotechnology Advances*, (April 8):E–Publication, 2011.

[17] R L Fahrner, H L Knudsen, C D Basey, W Galan, D Feuerhelm, M Vanderlaan, and G S Blank. Industrial purification of pharmaceutical antibodies Development, operation and validation of chromatography processes. *Biotechnology & Genetic Engineering Review*, 18:301–327, 2001.

[18] GE Healthcare. MabSelect SuRe - Studies on ligand toxicity, leakage, removal of leached ligand and sanitization. Application Note 11-0011-64 AA, 2004.

[19] S Sommerfeld and J Strube. Challenges in biotechnology production - generic processes and process optimisation for monoclonal antibodies. *Chemical Engineering and Processing*, 44:1123–1137, 2005.

[20] GE Healthcare. Two-step purification of monoclonal IgG1 from CHO cell culture supernatant. Application Note 28-9078-92 AA, 2007.

[21] GE Healthcare. MabSelect. Data File 18-1149-94 AE, 2008.

[22] J J Stickel and A Fotopoulos. Pressure-flow relationships for packed beds of compressible chromatography media at laboratory and production scale. *Biotechnology Progress*, 17(4):744–751, 2001.

[23] R Tran, J R Joseph, A Sinclair, D G Bracewell, Y Zhou, and N J Titchener-Hooker. A framework for the prediction of scale-up when using compressible chromatographic packings. *Biotechnology Progress*, 23(2):413–422, 2007.

[24] B Kelley, S A Tobler, P Brown, J L Coffman, R Godavarti, T Iskra, M Switzer, and S Vunnum. Weak partitioning chromatography for anion exchange purification of monoclonal antibodies. *Biotechnology and Bioengineering*, 101(3):553–566, 2008.

[25] G Jagschies. Where is biopharmaceutical manufacturing heading? *BioPharm International*, October 2008.

[26] R Hahn, P Bauerhansl, K Shimahara, C Wizniewski, A Tscheliessnig, and A Jungbauer. Comparison of protein A affinity sorbents ii. mass transfer properties. *Journal of Chromatography A*, 1093:98–110, 2005.

[27] D Low, R O'leary, and N J Pujar. Future of antibody purification. *Journal of Chromatography B*, 848(1):48–63, 2007.

[28] S Ghose, D Nagrath, B Hubbard, C Brooks, and S M Cramer. Use and optimization of a dual-flowrate loading strategy to maximize throughput in Protein-A affinity chromatography. *Biotechnology Progress*, 20:830–840, 2004.

[29] T M Przybycien, N S Pujar, and L M Steele. Alternative bioseparation operations life beyond packed-bed chromatography. *Current Opinion in Biotechnology*, 15:469–478, 2004.

[30] J Persson, D C Andersen, and P M Lester. Evaluation of different primary recovery methods for E.coli-derived recombinant human growth hormone and

compatibility with further down-stream purification. *Biotechnology and Bioengineering*, 90(4):442–451, 2005.

[31] J Persson, L Nystrom, H Ageland, and F Tjerneld. Purification of recombinant proteins using thermoseparating aqueous two-phase system and polymer recycling. *Journal of Chemical Technology and Biotechnology*, 74:238–243, 1999.

[32] C Kepka, J Rhodin, R Lemmens, F Tjerneld, and P E Gustavsson. Extraction of plasmid DNA from escherichia coli cell lysate in a thermoseparating aqueous two-phase system. *Journal of Chromatography A*, 1024:95–104, 2004.

[33] R M Banik, A Santhiagu, B Kanari, C Sabarinath, and S N Upadhyay. Technological aspects of extractive fermentation using aqueous two-phase systems. *World Journal of Microbiology and Biotechnology*, 19:337–348, 2003.

[34] A D Diamond and J T Hsu. Aqueous two-phase systems for biomolecule separation. *Advances in Biochemical Engineering and Biotechnology*, 47:89–135, 1992.

[35] S J Stocks and D E Brookes. Development of a general ligand for immunoaffinity partitioning in aqueous two-phase systems. *Analytical Biochemistry*, 173:86–92, 1988.

[36] I H Pan, H H Chiu, C H Lu, L T Lee, and Y K Li. Aqueous two-phase extraction as an effective toll for isolation of genopside from gardenia fruit. *Journal of Chromatography A*, 977:239–246, 2002.

[37] M Li, Z Q Zhu, and A E Rodrigues. Process integration of separation of amino acids by a temperature induced two phase system. *Industrial Engineering and Chemical Research*, 41:251–256, 2002.

[38] C Kepka, E Collet, J Persson, A Stahl, T Lagerstedt, F Tjerneld, and A Veide. Pilot-scale extraction of an intracellular recombinant cutinase from E.Coli cell homogenate using a thermoseparating aqueous two-phase system. *Journal of Biotechnology*, 103:165–181, 2003.

[39] M Li and T L Peeples. Purification of hyperthermophilic archael amylolytic enzyme (MJA1) using thermoseparating aqueous two-phase systems. *Journal of Chromatography B*, 807:69–74, 2004.

[40] F Hilbrig and R Freitag. Protein purification by affinity precipitation. *Journal of Chromatography B*, 790:79–90, 2003.

[41] N Labrou and Y D Clonis. The affinity technology in downstreama processing. *Journal of Biotechnology*, 36:95–119, 1994.

[42] H Stiborova, J Kostal, A Mulchandani, and W Chen. One step metal-affinity purification of histidine-tagged proteins by temperature-triggered precipitation. *Biotechnology and Bioengineering*, 82:605–611, 2003.

[43] M Eggert, T Baltes, F Garret-Flaudy, and R Freitag. Affinity precipitation - an alternative to fluidized bed adsorption? *Journal of Chromatography A*, 827:269–280, 1998.

[44] A Kumar, P O Wahlund, C Kepka, I Y Galaev, and B Mattiasson. Purification of histidine-tagged single chain Fv-antibody fragments by metal chelate affinity precipitation using thermoreponsive copolymers. *Biotechnology and Bioengineering*, 84:494–503, 2003.

[45] A Kumar, A A M Khalil, I Y Galaev, and B Mattiassono. Metal chelate affinity precipitation purification of (His)6-tagged lactate dehyrdrogenase using poly(vinyl)limidazole-co-N-isopropylacrylamide) copolymers. *Enzyme and Microbial Technology*, 33:113–117, 2003.

[46] T Mori, D Umeno, and M Maeda. Sequence-specific affinity precipitation of oligonucleotide using poly-(N-isopropylacrylamide)-oligonucleotide conjugate. *Biotechnology and Bioengineering*, 72(3):261–268, 2001.

[47] M A Taipa, R H Kaul, B Mattiasson, and J M S Cabral. Recovery of a monoclonal antibody from hybridoma cell culture supernatant by affinity precipitation using Eudragit S-100. *Bioseparation*, 9:291–298, 2001.

[48] J Kostal, A Mulchandani, and W Chen. Affinity purification of plasmid DNA by temperature triggered precipitation. *Biotechnology and Bioengineering*, 85:293–297, 2004.

[49] C Dennison and R Lovrien. Three phase partitioning: Concentration and purification of proteins. *Protein Expression and Purification*, 11:149–161, 1997.

[50] S Jain, R Singh, and M N Gupta. Purification of recombinant green fluorescent protein by three-phase partitioning. *Journal of Chromatography A*, 1035:83–86, 2004.

[51] B J A Paule, R Meyer, L F Moura-Costa, R C Bahia, R Carminati, L F Regis, V L C Vale, S M Freire, I Nascimento, R Schaer, and V Azevedo. Three-phase partitioning as an efficient method for extraction/concentration of immunoreactive excreted-secreted proteins of corynebacteriumpseudotuberculosis. *Protein Expression and Purification*, 34:311–316, 2004.

[52] A Sharma and M N Gupta. Three phase partitioning as a large-scale separation method for purification of a wheat germ bifunctional protease/amylase inhibitor. *Process Biochemistry*, 37:193–196, 2001.

[53] S Sharma and Gupta M N. Purification of phospholipase D from dacus carota by three-phase partitioning and its characterisation. *Protein Expression and Purification*, 21:310–316, 2001.

[54] S Sharma and M N Gupta. Purification of pectinases by three-phase partitioning. *Biotechnology Letters*, 23:1625–1627, 2001.

[55] A Sharma, K Mondal, and N Gupta. Separation of enzymes by sequential macroaffinity ligand-facilitated three phase partitioning. *Journal of Chromatography A*, 995:127–134, 2003.

[56] A Sharma and M N Gupta. Macroaffinity ligand-facilitated three phase partitioning (MLFTPP) for purification of xylanase. *Biotechnology and Bioengineering*, 80(2):228–232, 2002.

[57] K Mondal, A Sharma, and M N Gupta. Macroaffinity ligand-facilitated three phase paritioning (MLFTTP of alpha-amylase using a modified alginate. *Biotechnology Progress*, 19:493–494, 2003.

[58] K Mondal, A Sharma, and M N Gupta. Macroaffinity ligand-facilitated three-phase partitioning for purification of glucoamylase and pullulanase using alginate. *Protein Expression and Purification*, 28:190–195, 2003.

[59] C Jacobsen, J Garside, and M Hoare. Nucleation and growth of microbial lipase crystals from clarified concentrated fermentation broths. *Biotechnology and Bioengineering*, 57:666–675, 1998.

[60] R A Judge, M R Johns, and E T White. Protein purification by bulk crystallisation The recovery of ovalbumin. *Biotechnology and Bioengineering*, 48:316–323, 1995.

[61] N S Tavare and J Garside. Simultaneous estimation of crystal nucleation and growth kinetics from batch experiments. *Chemical Engineering Research and Design*, 64:109–118, 1986.

[62] T S Lee, J D Vaghjiani, G J Lye, and M K Turner. A systematic approach to the large scale production of protein crystals. *Enzyme and Microbial Technology*, 26:582–592, 2000.

[63] J Brange. Galenics of insulin. Springer-Verlag, Berlin, 1987.

[64] C Christy, G Adams, R Kuriyel, G Bolton, and A Seilly. High-performance tangential flow filtration: a highly selective membrane process. *Desalination*, 144:133–142, 2002.

[65] R van Reis, J M Brake, J Charkoudian, D B Burns, and A L Zydney. High-performance tangential flow filtration using charged membranes. *Journal of Membrane Science*, 159:133–142, 1999.

[66] M F Ebersold and A L Zydney. Separation of protein charge variants by ultrafiltration. *Biotechnology Progress*, 20:543–549, 2004.

[67] M F Ebersold and A L Zydney. The effect of membrane properties on the separation of protein charge variants using ultrafiltration. *Journal of Membrane Science*, 243:379–388, 2004.

[68] J H Vogel, B Anspach, K-H Kroner, J M Piret, and C Haynes. Controlled shear affinity filtration (CSAF) A new technology for integration of cell separation and protein isolation from mammalian cell cultures. *Biotechnology and Bioengineering*, 78(7):806–814, 2002.

[69] L R Castilho and F B Anspach. CFD-aided design of a dynamic filter for mammalian cell separation. *Biotechnology and Bioengineering*, 83(5):514–524, 2003.

[70] J H Vogel and K H Kroner. Controlled shear filtration: a novel technique for animal cell separation. *Biotechnology and Bioengineering*, 63:663–674, 1999.

[71] S R Shepard, G A Boyd, and J L Schrimsher. Routine manufacture of recombinant proteins using expanded bed adsorption chromatography. *Bioseparation*, 10:51–56, 2001.

[72] S R Shepard, R Boucher, J Johnston, R Boemer, G Koch, J W Madsen, D Grella, B K L Sim, and L Schrimsher. Large scale purification of recombinant human angiostatin. *Protein Expression and Purification*, 20:216–227, 2000.

[73] C Johansson, H J annd Jagersten and J Shiloach. Large scale recovery and purification of periplasmic recombinant protein from E.Coli using expanded bed adsorption chromatography followed by new ion exchange media. *Journal of Biotechnology*, 48:9–14, 1996.

[74] J R McDonald, M Ong, C Shen, Z Parandoosh, B Sosnowski, S Bussell, and Houston L L. Large-scale purification and characterisation of recombinant fibroblast growth factor-saporin mitotoxin. *Protein Expression and Purification*, 8:97–108, 1996.

[75] J Hubbuch, J Thömmes, and M R Kula. Biochemical engineering aspects of expanded bed adsorption. *Advances in Biochemical Engineering and Biotechnology*, 92:101–123, 2005.

[76] R Hjorth. Expanded-bed adsorption in industrial bioprocessing recent developments. *Trends in Biotechnology*, 15(6):230–235, 1997.

[77] A Jungbauer and R Hahn. Monoliths for fast bioseparation and bioconversion and their applications in biotechnology. *Journal of Separation Science*, 27:767–778, 2004.

[78] M Barut, A Podgornik, P Brne, and A Strancar. Convective interaction media short monolithic columns: Enabling chromatographic supports for the separation and puriifcation of large biomolecules. *Journal of Separation Science*, 28:1876–1892, 2005.

[79] L G Berruex, R Freitag, and T B Tennikova. Comparison of antibody binding to immobilized group specific affinity ligands in high performance monolith affinity chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, 24:95–104, 2000.

[80] A E Rodrigues, J C Lopes, Z P Lu, J M Loureiro, and M M Dias. Importance of intraparticle convection in the performance of chromatographic processes. *Journal of Chromatography A*, 590:93–100, 1992.

[81] A E Rodrigues, Z P Lu, J M Loureiro, and G Carta. Peak resolution in linear chromatography: effects of intraparticle convection. *Journal of Chromatography A*, 653:189–198, 1993.

[82] J Hagedorn, C Kasper, R Freitag, and T Tennikova. High performance flow injection analysis of recombinant Protein G. *Journal of Biotechnology*, 69:1–7, 1999.

[83] A Podgornik, J Jancar, M Merhar, S Kozamernik, D Glover, K Cucek, M Barut,

and A Strancar. Large-scale methacrylate monolithic columns design and properties. *Journal of Biochemical and Biophysical Methods*, 60:179–189, 2004.

[84] P Ardvission, F M Plieva, V I Lozinsky, I Y Galaev, and B Mattiasson. Direct chromatographic capture of enzyme from crude homogenate using immobilized metal affinity chromatography on a continuous supermacroporous adsorbent. *Journal of Chromatography A*, 986:275–290, 2003.

[85] M Bencina, A Podgornik, and A Strancar. Characterization of methacrylate monoliths for purification of DNA molecules. *Journal of Separation Science*, 27:801–810, 2004.

[86] J Urthaler, R Schlegl, A Podgornik, A Strancar, A Jungbauer, and R Necina. Application of monoliths for plasmid dna purification - development and transfer to production. *Journal of Chromatography A*, 1065:93–106, 2005.

[87] K Branovic, D Forcic, J Ivancic, A Strancar, M Barut, T Kosutic-Gulija, R Zgorelec, and R Mazuran. Application of short monolithic columns for improved detection of viruses. *Journal of Virological Methods*, 110:163–171, 2003.

[88] S L Williams, M E Eccleston, and N K H Slater. Affinity capture of a biotinylated retrovirus on macroporous monolithic adsorbents Towards a rapid single-step purification process. *Biotechnology and Bioengineering*, 89(7):783–787, 2005.

[89] R Ghosh. Protein separation using membrane chromatography: opportunities and challenges. *Journal of Chromatography A*, 952:13–27, 2002.

[90] C Charcosset. Purification of proteins by membrane chromatography. *Journal of Chemical Technology and Biotechnology*, (71):95–110, 1998.

[91] C Charcosset. Membrane processes in biotechnology. *Biotechnology Advances*, 24:482–492, 2006.

[92] J X Zhou and T Tressel. Basci concepts in Q membrane chromatography for large-scale antibody production. *Biotechnology Progress*, 22:341–349, 2006.

[93] Schaeffer R L, Mendenhall W, and Ott L. *Elementary Survey Sampling Fifth Edition*, page 95. Duxbury Press Boston, 1996.

[94] Levy P S and Lemeshow S. *Sampling of Populations: Methods and Applications, 4th Edition*, page 62. Wiley, 2008.

[95] PhRMA. Medicines in development. Biotechnology Report, 2008.

[96] Bioworld. Market-leading biotechnology drugs: Blockbuster dynamics in an ailing economy, 2009.

[97] F Wilcoxon. Individual comparisons by ranking methods. *Biometrics Bulletin*, 1:80–83, 1945.

[98] H B Mann and D R Whitney. On a test of whether one of two random variables is stochastically larger than the other. *Annals of Mathematical Statistics*, 18:50–60, 1947.

[99] R van Reis. Tangential flow filtration process and apparatus. U.S. Patent 5,256,294, 1993.

[100] R van Reis. Tangential flow filtration process and apparatus. U.S. Patent 5,490,937, 1993.

[101] J Peters, T Minuth, and W Schröder. Implementation of a crystallization step into the purification process of a recombinant protein. *Protein Expression and Purification*, 39:43–53, 2005.

[102] B D Kelley. Bioprocessing of therapeutic proteins. *Current Opinion in Biotechnology*, 12:173–174, 2001.

[103] M Rosen. The complexities, cost of midwest biotech drug development, wiscosin technology network. `http://wistechnology.com/article.php?id=377`, 2003.

[104] K Monal, S Jain, S Teotia, and S Gupta. Emerging options in protein bioseparation. *Biotechnology Annual Reiew*, 12:1–29, 2006.

[105] L Virine and M Trumper. Project decisions: The art and science. Vienna: Management Concepts, 2007.

[106] S S Farid, , J Washbrook, and N J Titchener-Hooker. Combining multiple quantitative and qualitative goals when assessing biomanufacturing strategies under uncertainty. *Biotechnology Progress*, 21(4):1183–1191, 2005.

[107] S H Zanakis, A Soloman, N Wishart, and S Dublish. Multi-attribute decision making A simulation comparison of select methods. *European Journal of Operational Research*, 107(3):507–529, 1998.

[108] R K Sinnott. *Coulson & Richardson's Chemical Engineering 3rd Edition*, volume 6, chapter Chemical Engineering Design, pages 242–282. Butterworth Heinemann, 1999.

[109] A I Jion, L-T Goh, and S K W Oh. Crystallisation of IgG1 by mapping its liquid-liquid phase separation curves. *Biotechnology and Bioengineering*, 95(5):911–918, 2006.

[110] B A Andrews, S Nielsen, and J A Asenjo. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. *Bioseparation*, 6:303 – 313, 1996.

[111] A M Azevedo and P A J Rosa. Optimisation of aqueous two-phase extraction of human antibodies. *Journal of Biotechnology*, (132):209–217, 2007.

[112] R van Reis. Charged filtration membranes and uses therefor. U.S. Patent 7,153,426 B2, 2006.

[113] Y Zhang and P S Cremer. Interactions between macromolecules and ions the hofmeister series. *Current Opinion in Chemical Biology*, 10:658–663, 2006.

[114] M Böstrom, Tavares F W, S Finet, F Skouri-Panet, A Tardieu, and B W Ninham. Why forces between proteins follow different hofmeister series for pH above and below pI. *Biophysical Chemistry*, 117:217–224, 2005.

[115] R van Reis and A Zydney. Membrane separations in biotechnology. *Current Opinion in Biotechnology*, 12:209–211, 2001.

[116] R van Reis and S Saksena. *Journal of Membrane Science*, 129:19–29, 1997.